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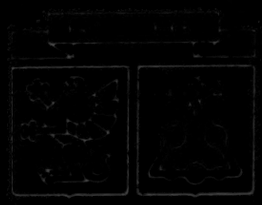

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ESTIMATION OF CARBOHYDRATES OF THE GLYCOLIPIDS IN TISSUES AND BODY FLUIDS

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The complexity and diverse composition of glycolipids, as well as their variable distribution in animal tissues, is not yet satisfactorily reflected in those estimation technics which employ determination of sugar, liberated by acid hydrolysis. Thus, the methods hitherto described estimate cerebrosides only. Extraction technics incorporated into these methods, however, dissolve all the lipids and glycolipids present in tissues and not only cerebrosides. If a lipid mixture, so obtained, is hydrolysed, all the lipid-bound carbohydrate will be estimated subsequently, irrespective of whether it originated from cerebrosides or gangliosides.

The application of the commonly used multiplication factor of 4.55, for the calculation of cerebrosides by their sugar content, appears to be of an equally doubtful value, when it is used indiscriminately on materials derived from tissues which may contain glycolipids other than certain cerebrosides. Glycolipids, besides containing not only galactose, may have greatly varying carbohydrate contents.

The distribution and exact nature of glycolipids in tissues is not readily assessed, without doing a thorough chemical investigation. Extreme variations may occur in pathological conditions. Although separation of a crude cerebroside mixture from other lipids is described within an estimation technic by RADIN, LAVIN AND BROWN[1], isolation of individual cerebrosides and gangliosides from a lipid mixture is still associated with great difficulties and uncertainties. Therefore, the method which is described in the present paper, estimates the total lipid-bound carbohydrate in tissues and body fluids and no attempt is made here to fractionate glycolipid mixtures or estimate individual glycolipids. The technic employed is a modification of a method, described earlier[2, 3], for the estimation of cerebrosides in blood.

It soon became obvious, when doing preliminary experiments, that several sources of error exist. Hence, it was decided to investigate the procedure and the nature of the errors in order to find measures for their elimination, whenever possible. An investigation was carried out accordingly by the following steps of the general procedure:

(a) extraction of lipid by organic solvents, (b) removal of interfering substances before and/or after hydrolysis, (c) hydrolysis of lipid and (d) estimation of the liberated sugar. These steps are common to most recent methods, which are based on the estimation of sugar, liberated from glycolipids by acid hydrolysis.

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EXPERIMENTAL

Reagents and materials

All reagents and organic solvents used throughout this work were of analytical purity. The orcinol and sulphuric acid reagents were prepared as described earlier[4]. Phrenosine (cerebron) was prepared from protagon and purified by the method of ROSENHEIM[5]. Protagon was prepared from sheep brain according to KITAGAWA AND THIERFELDER[6]. Methoxy-neuraminic acid was kindly supplied by Prof. E. KLENK.

Apparatus

The orcinol–sulphuric acid reaction and Bial's test of neuraminic acid were measured on a Unicam SP600 spectrophotometer. The colour reactions produced in the Somogyi–Nelson method were estimated by using a Spekker photoelectric absorptiometer with Ilford spectrum filter No. 608 (red). All measurements were made in 1-cm cells.

Procedure

Material, in amounts of 0.2 to 1.5 g, is ground, in an efficient tissue grinder, with 2.5 to 5 ml water to a fine suspension. Formalin-preserved materials or tough tissues may preferably be cut into thin sections (7μ) with a freezing microtome, previous to grinding, according to the suggestion of BRANTE[7]. A portion of the suspension, measured by weight, is used to determine the dry weight.

In a centrifuging tube of suitable size, 1 to 4 g of the suspension (or body fluids without pretreatment) is mixed with 15 to 20 times its volume of absolute ethanol. The mixture is brought to boiling in a water bath and centrifuged. The supernatant fluid is decanted into a beaker and the extraction is repeated twice with (5 to 10 times its volume of) boiling absolute ethanol and chloroform (3 : 1). These two extracts are added to the first.

The pooled extracts are evaporated to dryness at 80°, followed by reextraction of the lipids with 4 ml of a mixture of chloroform and methanol (9 : 1). After 20 min the extract is centrifuged and transferred, with two subsequent washings (3 ml each) of the same solvent, into a 25-ml glass-stoppered centrifuging tube. The extract should be clear, *i.e.* without any turbidity or cloudiness.

The contents of the centrifuging tube are vigorously shaken for 3 min with 10 ml of a 2% solution of trichloracetic acid in water to remove water-soluble interfering substances. The emulsion which forms is broken up by centrifugation and the clear trichloracetic-acid layer is carefully removed and discarded. Treatment with trichloracetic acid is repeated twice. The chloroform, containing the lipids, is turbid after this treatment, and may be cleared by the addition of some methanol. However, it is preferable to evaporate the solvent and redissolve the lipid in a few ml of chloroform–methanol (3 : 1).

The chloroform–methanol solution, containing the purified lipid, is quantitatively transferred (2 washings) to a 5- to 10-ml ampule and the solvent is evaporated. According to the amount of lipid to be analysed, 0.5- to 1.0-ml of water (or 0.1% solution of sodium lauryl sulphate in water) is added and an emulsion is formed by swirling the ampule in a hot water bath. Care should be taken not to lose water by evaporation during this operation. The emulsified lipid is mixed with the same amount

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(0.5 to 1.0 ml) of 5 *N* hydrochloric acid and the ampule sealed. The lipids are then hydrolysed in a boiling water bath for 45 min, shaking the ampule vigorously and repeatedly during the last 20 min. After cooling for a few minutes, in running tap water, the ampule is opened and the contents are transferred to a centrifuging tube in which they are shaken with chloroform, taking 7 ml of chloroform for each ml of hydrolysate. After centrifuging, an aliquot (usually half) of the clear supernatant

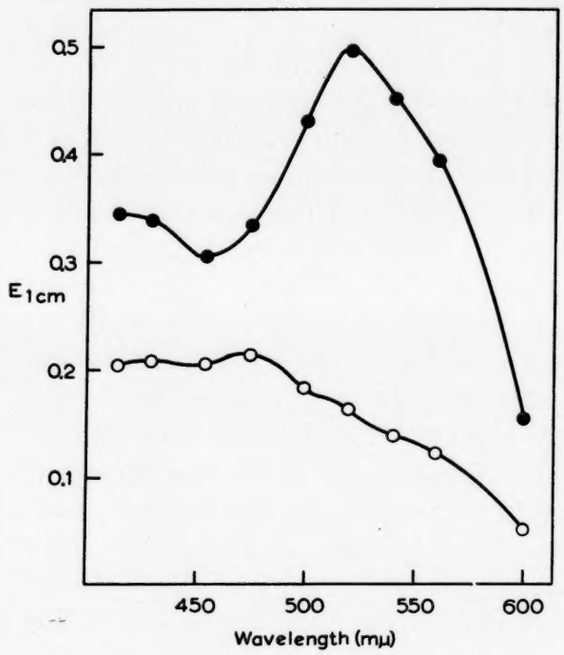

Fig. 1. Absorption spectra of glucose (○) and galactose (●) solutions (0.1 mg/ml) in the orcinol–sulphuric acid reaction. Colours were measured in a Unicam SP600 spectrophotometer (in 1-cm cells) against a reagent blank.

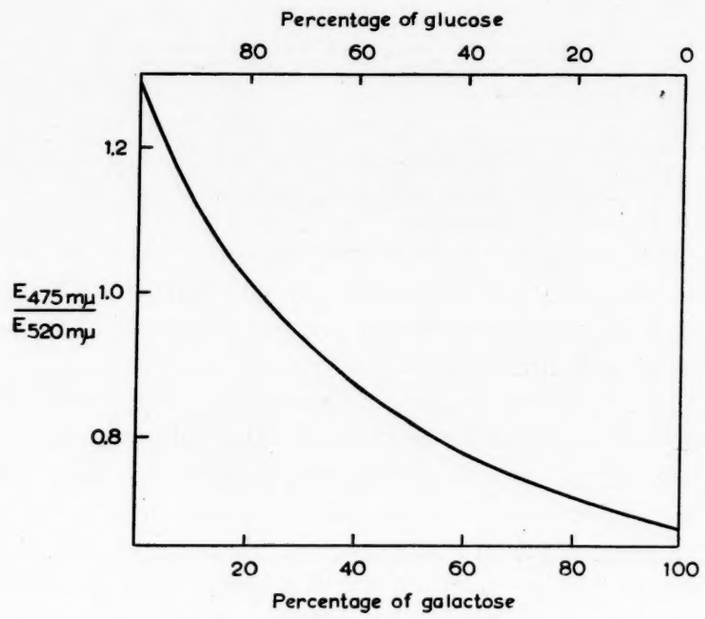

Fig. 2. Curve for the estimation of the proportions of glucose and galactose in mixtures of the two sugars. The curve has been calculated from extinction values measured at 475 and 520 mμ and given in Fig. 1.

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fluid is measured by a pipette, spread on the bottom of a 50-ml beaker and evaporated to dryness in a vacuum desiccator, containing H_2SO_4 and NaOH pellets[8]. Evaporation and absorption of HCl is complete within 1 hour. The contents of the beaker are finally redissolved in a known volume of water and the sugar content of the solution is estimated either by the improved orcinol–sulphuric acid method[4] or by using the copper reagent of SOMOGYI[9]. Carbohydrate content of the solution to be analysed by the orcinol–sulphuric acid method should be between 0.05 and 1.0 mg/ml approximately.

It is advisable to measure the colour, obtained in the orcinol reaction, against a blank. For this purpose 10 ml 31.2 *N* sulphuric acid is added to 1.25 ml of the carbohydrate solution in a 100-ml flask, with cooling. From this mixture a 4.5-ml portion is measured into each of two flasks, the one containing 0.5 ml water (I), the other 0.5 ml of the orcinol reagent (II) and mixed with cooling. The mixtures are then treated as has been previously described[4], reading II against I in a spectrophotometer or other suitable instrument.

The blank reading due to the orcinol must be estimated separately[4]. Once determined, this value can be used in all subsequent calculations being simply subtracted from the value obtained for the hydrolysate.

The absorption spectrum of the colour reaction with orcinol and sulphuric acid reveals the nature of the carbohydrate in the hydrolysate (Fig. 1). The proportions of galactose and glucose, if they occur together in the hydrolysate, may be calculated in the same manner as is usual in the spectrochemical analysis of a two-component mixture. Measurements are preferably made at the maxima of glucose (475 mμ) and galactose (520 mμ). The curve in Fig. 2 facilitates such a calculation. Using the ratio of the two readings ($E_{475\ m\mu}/E_{520\ m\mu}$) the proportions of galactose and glucose in any unknown mixture may be read directly from the graph. Once the proportions of the two carbohydrates in the mixture are known, the actual amount of the carbohydrate may be calculated, as follows:

$$\text{galactose (mg/ml)} = \frac{\dfrac{E_{(\text{gal})\ 520}\, n\% \times E_{(\text{test})\ 520}}{E_{(\text{gal})\ 520}\, n\% + E_{(\text{gluc})\ 520}\,(100-n\%)}}{E_{(\text{gal})\ 520}} \times 0.1$$

$$= \frac{E_{(\text{test})\ 520}\, n\%}{E_{(\text{gal})\ 520}\, n\% + E_{(\text{gluc})\ 520}\,(100-n\%)} \times 0.1$$

A similar formula may be used for glucose in evaluating the extinction value measured at 475 mμ. For the quantitative estimation of carbohydrate, when only galactose or only glucose is present, measurement of the extinction at but one wavelength is required, at 520 or at 475 mμ respectively.

NELSON's arsenomolybdate reagent[10] was used in estimating the carbohydrate in connection with Somogyi's copper reagent. The colours were read 10 min after the addition of the arsenomolybdate reagent. During this time the mixtures were repeatedly shaken and finally water was added to a volume of 10 ml. 1 ml of the sugar-containing solution was used with 1 ml of each of the two reagents. The orcinol reaction or yeast fermentation[11] may be used to determine proportions of galactose and glucose when they occur in mixture. Reduction values found for glucose and galactose compare as 1 to 0.785.

The hydrolysate, after further purification, is suitable for sugar analysis using

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paper partition-chromatographic technics. It may be defatted with ether, desalted with pyridine, spotted (approximately 0.05 mg sugar) on to Whatman No. 1 paper and run as a descending chromatogram with *n*-butanol–pyridine–water, 5 : 3 : 1. (J. V. DUNCKLEY, unpublished data).

Investigation of errors and of validity of method

Extraction of glycolipids. Materials (mainly spleen and brain) which had been extracted with ethanol and ethanol–chloroform were collected, dried and ground to a fine powder. The powder (1.5606 g) was extracted with chloroform–methanol (3 : 1) for 9 h in a Soxhlet apparatus. After evaporation of the solvent, the brown residuum, remaining in the flask, was dissolved in chloroform–methanol (9 : 1), treated with trichloracetic acid in a centrifuging tube and hydrolysed as described under *Procedure*. No carbohydrate could be detected in the hydrolysate by the orcinol–sulphuric acid reaction. Thus, the extraction of glycolipids from tissues, as described in the procedure, appears to be satisfactorily complete.

Effect of trichloracetic acid treatment on lipid solutions. Trichloracetic acid treatment of the lipid solution was proposed in a previous communication[3] for the removal of water soluble substances, which could interfere with the estimation of liberated carbohydrates in later stages of the operation.

FAWAZ, LIEB AND ZACHERL[12] were the first who employed trichloracetic acid solutions in lipid analysis. These authors treated the prepared tissues with a watery solution of trichloracetic acid, in order to remove the non lipid-bound carbohydrates, before the extraction of the lipids. It was found, on experimenting with this technic, that some of the tissue proteins, treated with trichloracetic acid, became soluble in alcohol, used for the extraction of the lipids. Therefore, a treatment with trichloracetic acid of the lipid extract itself has been recommended[3]. This technic gave satisfactory results and has since been adopted by OTTENSTEIN, SCHMIDT AND THANNHAUSER[11] and UZMAN[13].

Since gangliosides are soluble in water[14], treatment with an aqueous solution of trichloracetic acid might lead to some loss of these lipids during the estimation. An investigation of this potential source of error appeared, therefore, desirable.

The behaviour of gangliosides on such treatment was investigated by the aid of neuraminic (sialic) acid determinations using the method of KLENK AND LANGERBEINS[15]. Since no purified ganglioside preparation was available, an extract of grey matter from the frontal lobe of a human brain was used.

The grey matter was extracted with ethanol and ethanol–chloroform, as described and then reextracted with chloroform–methanol (4 : 1). The solution was subdivided into twenty 2-ml aliquots, which were transferred to centrifuging tubes. The solvent was evaporated. The neuraminic acid content was estimated on two of the samples without further treatment. Remaining samples (in centrifuging tubes) were treated in duplicates. The samples were dissolved in chloroform–methanol. 3-ml portions of the solvent mixture were added, but containing varying proportions of chloroform and methanol, to each of the tubes carried in duplicate. All tubes were treated 3 times with 3-ml portions each of a 2% solution of trichloracetic acid in water by shaking the contents vigorously for 3 min. Both, the organic phase and the collected washing fluids were evaporated and the residua saved for neuraminic acid estimations.

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Only 89.0% of the gangliosides (estimated by the neuraminic acid contained in them) could be recovered, when the lipid was dissolved in a 4 : 1 mixture of chloroform with methanol; 11.0% of the lipid had been lost to the trichloractetic acid washing fluid. Dissolving the lipid in a 9 : 1 mixture of chloroform with methanol, before treatment, improved recoveries, 96.1% of the gangliosides being found in the evaporated organic phase and only 3.9% in the trichloracetic acid solution.

Evidence was found that there is a pentose-like substance amongst the interfering compounds which are removed by the trichloracetic acid treatment. Thus, upon performing Bial's test on the untreated lipid samples, the characteristic reaction of neuraminic acid was observed which masked a green colour reaction, developing

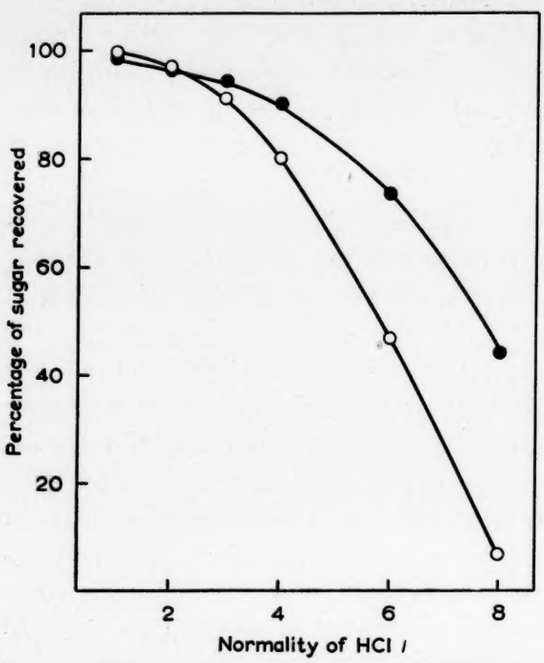

Fig. 3. Effect of concentration of HCl on glucose (○) and galactose (●) at 100°; time of heating 45 min. Sugars were estimated by Somogyi's method.

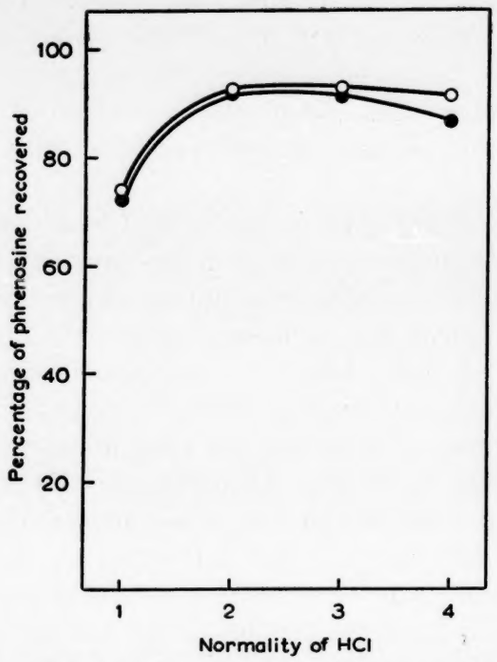

Fig. 4. Effect of concentration of HCl on the hydrolysis of phrenosine at 100°; time of heating 45 min. Sugar released was estimated by Somogyi's method (●) and by the orcinol–sulphuric acid reaction (○).

simultaneously. Two maxima were found, one at 572 mμ (neuraminic acid) and the other at 660 mμ (interfering substance). This is consistent with the observations of KLENK AND LANGERBEINS[15]. Such an interfering green colour reaction did not occur when the lipid solution had been treated with trichloracetic acid, but it appeared in the trichloracetic acid washing fluid. In Bial's test the absorption spectrum of the green colour reaction is similar to that of D-ribose; the absorption maxima are identical (660 mμ).

Similar observations were made with the orcinol–sulphuric acid reaction. Superimposed on the colour reactions of lipid-bound carbohydrates in extracts from brain and spleen, *not treated* with trichloracetic acid, an aldopentose reaction was clearly distinguishable.

FISHER *et al.*[16], using the orcinol–sulphuric acid reaction, also found pentose-containing compounds, in appreciable quantities, in extracts of chicken brain.

To estimate the concentration of neuraminic acid in above experiments, the

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colour reactions and their absorption spectra were treated as two-component mixtures and calculated as such. This presumes that the interfering substance is identical with D-ribose.

Effect of acid treatment on glucose and galactose. Incomplete recovery of cerebrosides has been variously reported in the literature[17-20], although methods are described which claim a near 100% or 100% recovery[21, 22]. Little attention is paid in these latter works to the observation of LEVENE AND MEYER[17], who reported as early as 1917, that they could recover only 97.15% of galactose which was subjected to a treatment with 2% HCl at 100° for 4 h.

Decomposition of carbohydrates during the acid hydrolysis could at least partly explain incomplete recoveries of cerebrosides. A behaviour of glucose and galactose on acid treatment at 100° was, therefore, investigated.

Solutions of D-glucose and D-galactose (0.2 mg/ml), containing hydrochloric acid of the desired normality, were heated in sealed ampules at 100° (boiling water bath). After cooling, the ampules were opened and 0.5-ml portions of the solutions measured into and spread on the bottom of 50-ml beakers. These were evaporated to dryness in a vacuum desiccator over NaOH pellets and H_2SO_4. The dried contents of each beaker were then redissolved in 2 ml of distilled water and the sugar in aliquots was estimated.

TABLE I

EFFECT OF ACID TREATMENT ON GLUCOSE AND GALACTOSE*

Time of heating at 100° in min	*Percentage of sugars recovered*							
	2 N HCl				*3 N HCl*			
	Glucose		*Galactose*		*Glucose*		*Galactose*	
	S	*O*	*S*	*O*	*S*	*O*	*S*	*O*
15	100.2	98.7	98.9	100.0	96.0	100.2	97.3	99.0
30	99.4	99.6	97.8	100.0	93.2	98.1	95.4	97.3
45	96.7	98.2	96.2	99.2	91.0	96.7	94.3	97.1
60	95.0	98.5	95.4	97.9	87.4	98.3	90.2	96.8

* Sugars were estimated with Somogyi's method (S) and the orcinol–sulphuric acid reaction (O).

Two series of experiments were made. In one series the time of heating was kept constant (45 min) and the concentration of the acid varied, in the other, employing 2 and 3 *N* acids, the time of heating was varied.

The results, which are given in Fig. 3 and in Table I, indicate that recovery depends on both, acid concentration and time of heating. It may be noted, that even with acid concentrations of 2 and 3 *N* (which concentrations are used in most recent works), loss of carbohydrate, though small, is significant. Recoveries with the orcinol method were slightly better than with Somogyi's copper method.

Performing the acid treatment in sealed ampules or test tubes is preferable to open test tubes, as recoveries are better and the results more consistent.

Hydrolysis of phrenosine. Incomplete hydrolysis of glycolipids is another possible source of error which could be blamed for an unsatisfactory recovery of carbohydrates. Hence, the hydrolysis of phrenosine by HCl at 100° was studied to investigate this step in the procedure and to establish its optimum conditions.

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Phrenosine (dried *in vacuo* above sulphuric acid) was dissolved in chloroform–methanol (3 : 1) to give a 0.2% solution (w/v). 1-ml portions of the solution were measured into ampules and the solvent evaporated. The lipid was emulsified in 0.5 ml water, 0.5 ml of HCl of twice the normality finally required (see Figs. 4, 5 and 6) was added and the ampules sealed. It was then hydrolysed and treated further as described under *Procedure*. In calculating phrenosine recoveries, the galactose values were multiplied by 4.6.

As with previous experiments, the effect of acid concentration and time of heating on hydrolysis was studied in series to obtain information about optimal conditions.

Thus, in the first series, the concentration of HCl was varied, all samples of phrenosine being heated for 45 min at 100° (Fig. 4). Since best recoveries were obtained with 2 and 3 *N* acids, a second and third series was done in which phrenosine was hydrolysed

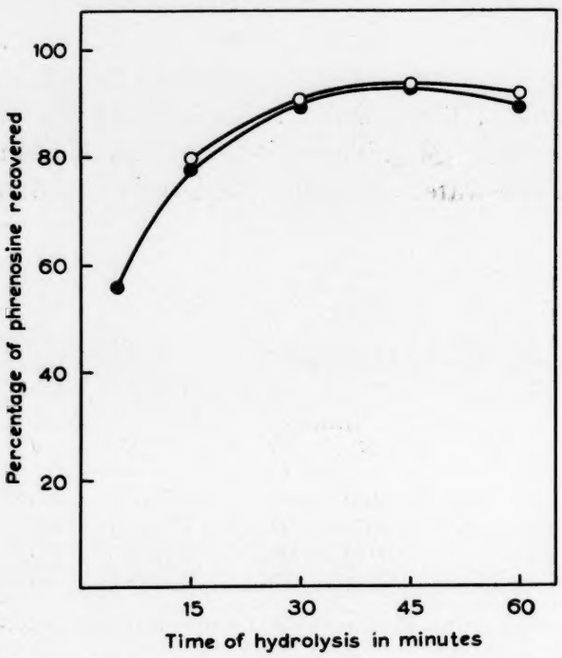

Fig. 5. Effect of time on hydrolysis of phrenosine by 2 *N* HCl at 100°. Sugar released was estimated by Somogyi's method (●) and by the orcinol–sulphuric acid reaction (○).

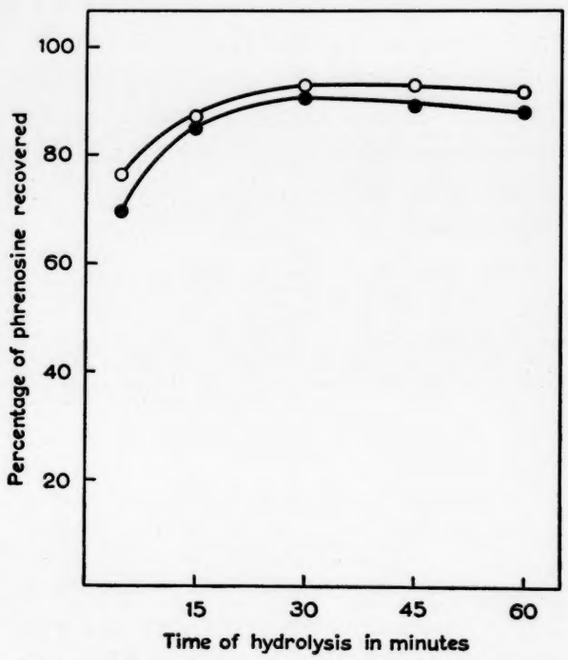

Fig. 6. Effect of time on hydrolysis of phrenosine by 3 *N* HCl at 100°. Sugar released was estimated by Somogyi's method (●) and by the orcinol–sulphuric acid reaction (○).

in 2 and 3 *N* acids, varying the time of heating (Figs. 5 and 6). All experimental points in the Figs. represent the mean value of three independent estimations.

These experiments showed that, although hydrolysis of phrenosine is more rapid using 3 *N* acid, somewhat less of the substance was recovered than when using 2 *N* acid. Since best recoveries were obtained with 2 *N* acid upon 45 min heating (92.5% with Somogyi's method and 93.0% with the orcinol test) and with 3 *N* acid upon 30 min heating (90.7% and 92.9% respectively), 2.5 *N* hydrochloric acid for 45 min was adopted as the standard.

Recovery of phrenosine added to serum lipids. Some of the experimental work, reported in the literature on the analysis of glycolipids, was done by using purified cerebroside preparations only. However, interference of lipids other than glycolipids,

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producing unspecific reactions is by no means a remote possibility. Hence, the effect of complex lipid mixtures on recoveries of added phrenosine was investigated.

Serum lipids were chosen as a natural lipid mixture, as it was shown that human serum does not contain measurable amounts of glycolipids[3, 11].

Pooled human serum was extracted as described under *Procedure*. After treatment with the trichloracetic-acid solution, the solvents were evaporated. The contents of the centrifuging tube were redissolved in chloroform–methanol (3 : 1) to give a solution of which 1 ml contained the lipids derived from 1.5 ml of human serum. 1-ml portions of this solution were measured into ampules. To each of 5 ampules 2 mg phrenosine was added (in solution). 3 ampules of serum lipid were used without the addition of phrenosine to serve as blanks. The solvent in all ampules was evaporated and the contents, after emulsification, were hydrolysed in 2.5 *N* hydrochloric acid (0.5 ml water–0.5 ml 5 *N* HCl) for 45 min and treated further as described.

It was observed, that the specimens which served as blanks, gave a small reduction value in Somogyi's method and a faint, yellowish colour in the orcinol test. These are probably non-specific reactions and would correspond (if calculated as sugar) to less than 0.01 mg lipid-bound carbohydrate/1.5 ml serum. In subsequent determinations, reported here, this value has been subtracted from the total where phrenosine–serum lipid mixtures were analysed. With this correction factor, recovery values for phrenosine mixed with serum lipids were: 89.5% to 92.8% (mean value: 91.4%) with the copper method and 90.6% to 96.0% (mean value: 92.9%) with the orcinol test. These values are consistent with those obtained on phrenosine alone and indicate no adverse effects of lipids on recoveries of added phrenosine.

Neuraminic acid in the orcinol–sulphuric acid reaction. In several investigations, related and non-related to the analysis of glycolipids, the orcinol reaction was found to possess a very high specificity for carbohydrates, which in the author's observations is greater than that shown by several other frequently used phenolic compounds, such as anthrone, thymol, carbazole and resorcinol.

KLENK AND LANGERBEINS[15], who described an estimation for neuraminic acid, used a colour reaction produced with orcinol, ferric chloride and hydrochloric acid (Bial's reagent). Thus a colour reaction of neuraminic acid with orcinol and sulphuric acid might be suspected. An investigation of this possibility appeared even more desirable as BRANTE[7], although not relying on experimental evidence, remarks that "...the orcine reaction may possibly be deleteriously influenced by the neuraminic acid in the gangliosides, thereby giving rise to errors in the determination of galactose- and glucose-containing lipids".

It was found that 1 ml of a methoxy-neuraminic acid solution (0.1 mg/ml) gave no appreciable colouration in the orcinol–sulphuric acid reaction, any absorption being mainly in the ultraviolet part of the spectrum. The extinction coefficient measured at 450 mμ was 0.008, the absorption gradually and completely disappearing, when moving further towards the red.

DISCUSSION

Extraction technics commonly used in the determination of cerebrosides (glycolipids) differ only little from those employed in lipid analysis and appear to be satisfactory.

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A more difficult question is the elimination of nonspecific reactions which impair the estimation of the lipid-bound carbohydrate. Determinations of the reducing power of a lipid extract before and after hydrolysis (in combination with zinc hydroxide precipitation) were thought to allow the estimation of lipid-bound carbohydrate only. This principle was introduced by KIMMELSTIEL[23] and subsequently developed by KIRK[21] and BRAND AND SPERRY[22].

In spite of the attractiveness of the principle, incorporated in these estimations, above methods may yield inconsistent results when applied to the determination of glycolipids in tissues or body fluids, as interference with reduction of lipid-bound carbohydrate is not satisfactorily prevented. Observations made on blood serum, in which no measurable amounts of glycolipids were found by THANNHAUSER and associates[11,24] and by BRÜCKNER[3], may support this view when contrasted with the findings of KIRK[25]. However, when lipids, derived from tissues with very high glycolipid contents (white matter of brain, etc.) are analysed, the error in the estimation of the lipid-bound carbohydrate may not be too great. Lipid-bound carbohydrate content of such materials is so high in comparison, that the effect of unspecific reactions is of a smaller consequence.

Investigations made earlier[3] have pointed to the necessity (a) of removing all *water-soluble* carbohydrates and/or interfering substances *previous* to hydrolysis and (b) of removing all *lipid-soluble* interfering substances and/or lipids *after* hydrolysis. The expected net result is the estimation of only lipid-bound carbohydrates. These requirements are fulfilled by (a) washing of the lipid extract with a solution of trichloracetic acid in water and by (b) an extraction of the hydrolysate with chloroform.

BRANTE[7] who studied the usefulness of washing the chloroform–methanol reextract with trichloracetic acid, found the extract cloudy after such a treatment. This can hardly be a disadvantage, since the cloudiness is caused by the trichloracetic acid treatment, which modifies the solvent properties of the organic phase. It readily clears on the addition of a small amount of methanol. Alternatively and preferably it may be eliminated by evaporating the solvent and redissolving the lipid. The effect of a treatment with trichloracetic acid on phospholipids has not been investigated, nor was such a treatment suggested by BRÜCKNER.

As far as can be ascertained, the only disadvantage of the trichloracetic acid treatment is a possible small loss of gangliosides. By choosing a convenient proportion of methanol and chloroform in the organic phase, this loss can be reduced to a minimum.

Although carbohydrates when heated to 100° are more stable in dilute acid than in alkaline or even neutral solutions, they are partly or completely destroyed by concentrated mineral acids. This is demonstrated in the data of Fig. 3, where the effect of treating glucose and galactose at 100° with HCl of varying normalities is illustrated.

In the experiments with phrenosine (Figs. 5 and 6) two principle sources of error may be observed. These are: (A) loss of carbohydrate due to the effect of acid during the hydrolysis at 100° (Fig. 3 and Table I) and (B) incomplete hydrolysis of the glycolipid. It would appear that increased time of heating could correct this. However, increased time of heating actually results in a decreased yield, because the gain from more complete hydrolysis is more than offset by loss of the carbohydrate itself. It is obvious that a compromise must be thought under these conditions, which is hydrolysis with 2.5 *N* HCl for 45 min at 100°.

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Thus, a significant error is inherent to the principle of acid hydrolysis and no full recovery of cerebrosides can be expected. Unsatisfactory treatment of the lipid material, previous to the estimation of the carbohydrate, may simulate in some methods full recoveries by nonspecific reactions, which modify the reduction value obtained on the lipid-bound carbohydrate. Any alteration in such methods of the very narrow range of experimental conditions, chosen for a 100% recovery, may then easily lead to considerable variations in recoveries[22].

Investigations pertinent to the hydrolysis of gangliosides were not performed as no ganglioside preparation was available. It must be concluded from the investigations of KLENK[14] (see also several other papers of KLENK and associates), that gangliosides, on conditions of the technic described, are hydrolysed similarly to cerebrosides.

The slightly better recovery of the acid-treated sugar with the orcinol–sulphuric acid reaction, in contrast to the copper method, is of interest. In the former test decomposition products of carbohydrates, treated with a concentrated mineral acid (H_2SO_4) react with orcinol to form coloured compounds[4]. It may be presumed, that on acid hydrolysis some of the carbohydrate is decomposed in such a way that its reducing power is decreased to a greater extent than its reactibility with orcinol. This circumstance is an advantage of the orcinol method in glycolipid analysis.

Neuraminic acid has no deleterious effect on the estimation of glucose and galactose, as no appreciable colour reaction could be observed with this substance in the orcinol–sulphuric acid test. Similar observations were made by BÖHM, DAUBER AND BAUMEISTER[26].

ACKNOWLEDGEMENTS

The author would like to express his gratitude to Professor E. KLENK (Cologne) for a gift of methoxy-neuraminic acid and to Professor H. C. HOPPS (Oklahoma City) for his help and advice in writing the text for the manuscript. His thanks should also be extended to Miss ELIZABETH A. MONTGOMERY for the preparation of the figures.

SUMMARY

A method is described for the quantitative estimation of carbohydrates in glycolipids of tissues and body fluids. The glycolipids are extracted from pretreated tissues and, after removing interfering substances, are hydrolysed. The carbohydrate released may be estimated either by Somogyi's copper reagent or by the orcinol–sulphuric acid reaction. Using the latter reaction, the nature of the carbohydrate can be determined and the proportions of glucose and galactose estimated, if they occur together in the glycolipids.

The average recovery of galactose in phrenosine is 93% with the orcinol–sulphuric acid reaction and 91% with the copper reagent of Somogyi. Factors responsible for this discrepancy as well as other steps in the procedure are investigated and discussed. Destruction of carbohydrate on acid treatment and incomplete hydrolysis of phrenosine were found to be the chief sources of error.

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NOTE ADDED IN PROOF

E. SVENNERHOLM AND L. SVENNERHOLM, *Scand. J. Clin. & Lab. Invest.*, 10 (1958) 97, propose a method for the estimation of "true" cerebrosides in plasma, in which they employ a partition technic and a modified orcinol reaction.

Their observations are not consistent with the opinion held in this paper and with findings reported in other communications[3, 11, 24]. Proof may be furnished by attempting to isolate from and identify the cerebrosides, if present, in human blood plasma and/or serum.

Added November 24th, 1958

SIMULTANEOUS DETERMINATION OF ISONIAZID AND *d*-CYCLOSERINE BY SODIUM PENTACYANOAMMINEFERROATE

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The colorimetric determmination of isoniazid (INH) in biological fluids and tissues by sodium pentacyanoammineferroate was described in previous papers [1-3]. The method was specific for the compound, the INH analogues being unable to give a chromogen. No investigation was carried on other antituberculous drugs, such as *para*-aminosalicylic acid, streptomycin, *d*-cycloserine.

Following requests from different research laboratories on the possible interference by these compounds, we carried out a new series of experiments, which resulted in the possibility of a simultaneous determination of *d*-cycloserine and INH in biological fluids.

The complete study of the reaction between *d*-cycloserine and sodium pentacyanoammineferroate, even in presence of INH, and the conditions of the quantitative assay are described in the present paper.

METHOD

Reagents

1. *d*-cycloserine (Roche) (*d*-4-amino-3-isoxazolidone), stock solution: 0.1 g of the pure compound is dissolved in water and diluted to 100 ml. To be stored in a refrigerator and discarded after a week. *2.* Isoniazid (Lepetit) (isonicotinoylhydrazide), stock solution: 0.1 g of the pure compound is dissolved in water and diluted to 100 ml. *3.* Metaphosphoric acid, HPO_3, 20%. *4.* Diammonium phosphate, $(NH_4)_2HPO_4$, 16.5%. *5.* Sodium pentacyanoammineferroate, $Na_3[Fe(CN)_5NH_3]$, 0.4% in 0.02 *N* NH_4OH. The preparation of this substance was previously described [1, 2].

Procedure

1 ml of fresh serum is added to 2 ml of distilled water in a centrifuge tube, followed by 1 ml of 20% metaphosphoric acid. The tube is shaken and allowed to stand at room temperature for 10 min, before centrifuging for the same period at 4000 r.p.m. The protein precipitate packs well and 2 ml of the clear supernatant are easily transferred to a Beckman cell or a colorimetric tube. 0.5 ml of diammonium phosphate and 0.5 ml of 0.4% sodium pentacyanoammineferroate are added, shaking after each addition. The contents of cells or tubes are thoroughly mixed by inverting, and after 10 min the intensity of the color is read at 430 (No. 42 filter), and at 630 mμ (No. 64 filter) against a blank prepared by substituting water for the sample. Results are referred to two calibration curves, which are checked with each set of determinations by running one or two standard samples.

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RESULTS

Absorption spectra of chromogens. While isoniazid develops a yellowish color within 5–10 min, which is stable at least for 20 min, *d*-cycloserine gives a quite different chromogen, whose absorption maximum is at 630 mμ. Fig. 1 shows the absorption spectrum of the reaction product between *d*-cycloserine and $Na_3[Fe(CN)_5NH_3]$, compared with the chromogen given by isoniazid. The former does not show an absorption spectrum at 470 mμ and shorter wavelengths, while the chromogen given by isoniazid does not absorb light at 630 mμ. As previously stated[4], the interaction between *d*-cycloserine and sodium pentacyanoammineferroate seems to require two molecules of the reagent for each molecule of the compound.

Stability of chromogens. The reaction between isoniazid and sodium pentacyanoammineferroate was fully investigated in previous papers[1–3]. The chromogen of *d*-cycloserine with the same compound shows its absorption maximum at 630 mμ, when the reaction is allowed to occur at a pH value between 5.5 and 6. The maximum is shifted towards the shorter wavelengths when the pH decreases; the reverse for higher pH values. The chromogen is stable for at least 1 h.

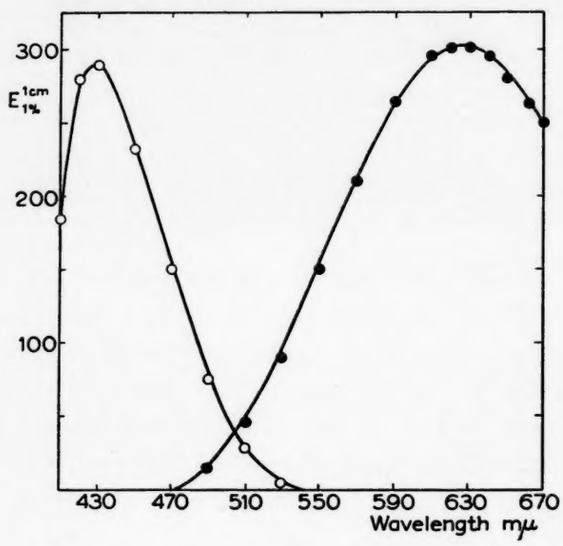

Fig. 1. Absorption spectra of INH (o—o) and *d*-cycloserine (●—●) chromogens at pH 5.7 in metaphosphoric acid–diammonium phosphate mixture. Abscissa: Wavelength in mμ; ordinate: $E^{1\,cm}_{1\,\%}$.

Influence of pH. The reaction rate between *d*-cycloserine and $Na_3[Fe(CN)_5NH_3]$ is related to the hydrogen-ion concentration. Fig. 2 shows that there is not a sharp optimal pH, but an optimal pH range between 4.7 and 6.3. It seems, indeed, that the most reactive form of *d*-cycloserine is the dipolar form which exists near neutrality, as shown by NEILANDS[5].

Calibration curve. Fig. 3 shows the direct proportion between the developed color and the *d*-cycloserine concentration. It should be kept in mind that, although the INH chromogen is quite different from that of *d*-cycloserine, a decrease in the optical density of the latter takes place in presence of high INH concentrations (see next

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section). In antituberculous therapy, however, the INH level in biological fluids very seldom exceeds that of *d*-cycloserine, so that practically no interference occurs.

Concentration of the reagent solution. In order to secure a full reaction of sodium pentacyanoammineferroate with both compounds, a 0.4% solution of the reagent was chosen. Such a concentration is sufficient, even when the sample contains 50 μg/ml of each drug.

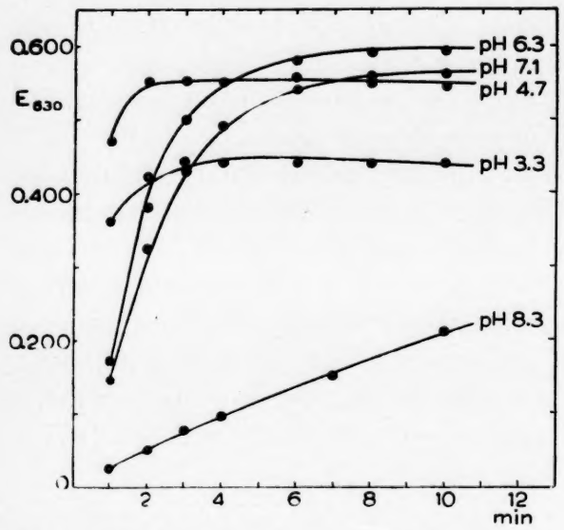

Fig. 2. Rate of the reaction between *d*-cycloserine and sodium pentacyanoammineferroate at different pH values, obtained with *McIlvaine* buffer. Abscissa: min; ordinate: E_{630}.

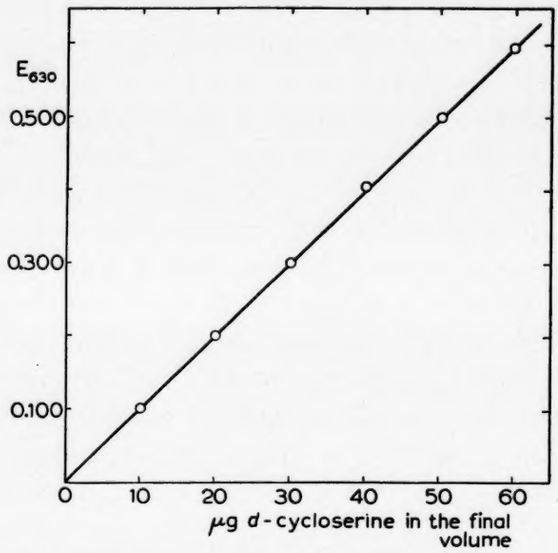

Fig. 3. Calibration curve of *d*-cycloserine standard solution (sample + water 1.5 ml; metaphosphoric acid 0.5 ml; diammonium phosphate 0.5 ml; reagent 0.5 ml). Abscissa: μg *d*-cycloserine in the final volume; ordinate: E_{630}.

Interference by other antituberculous drugs. In order to test possible interference by other antituberculous drugs which are currently associated in therapy, we have examined *para*-aminosalicylic acid and streptomycin. The former does not give any chromogen while streptomycin faintly reacts with sodium pentacyanoammineferroate giving a chromogen whose absorption maximum is at 490 mμ; its $E^{1\text{ cm}}_{1\%}$ is 1.9, and at 430 mμ the extinction is negligible.

TABLE I

RECOVERY OF *d*-CYCLOSERINE ADDED TO 1 ml BLOOD SERUM WITH OR WITHOUT INH

Sample	*INH added* μg	*d-cycloserine added* μg	*INH recovered* μg	%	*d-cycloserine recovered* μg	%
1	–	5	–	–	5	100
2	–	10	–	–	9.8	98
3	–	20	–	–	20.3	101.5
4	–	50	–	–	49.6	99.2
5	5	5	5.1	102	4.9	98
6	5	10	50	100	10	100
7	50	20	49.8	99.6	20.1	100.5
8	50	50	50	100	49.7	99.4

Recovery experiments. Table I summarizes recovery experiments which were carried out with serum by adding isoniazid and *d*-cycloserine in different combina-

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tions and in amounts ranging from 5–50 μg/ml. Standard deviations calculated on 10 determinations of 25 μg/ml of *d*-cycloserine alone and on 10 determinations of the same in presence of 25 μg/ml of INH are $\pm$ 0.26 and $\pm$ 0.29 respectively.

DISCUSSION

The present method is superior in respect to the other accepted methods for the determination of INH[1] and *d*-cycloserine[6], because it allows the simultaneous determination of both compounds in biological fluids. Although simple it is accurate and rapid, requiring no particular laboratory equipment furthermore, no physiological substances interfere with the colorimetric reaction, with the exception of nicotinamide, which represents an interfering agent only in the case of some tissues[2].

The method is very specific as regards other antituberculous drugs, as shown by our experiments with *para*-aminosalicylic acid and streptomycin. The most interesting feature of the reaction between *d*-cycloserine and $Na_3[Fe(CN)_5NH_3]$ is the interference of high INH concentrations, probably due to a displacement reaction. This hypothesis was tested by adding an excess of INH to the chromogen of *d*-cycloserine (*d*-cycloserine/$Na_3[Fe(CN)_5NH_3]$ ratio equal to 1 : 2) and by following the decrease of the optical density at 630 mμ. The decrease at 630 mμ seemed to run parallel to the optical density increase at 430 mμ. As previously stated, however, such an interference is not a limiting factor in practice.

SUMMARY

A colorimetric reaction for simultaneous determination of isoniazid and *d*-cycloserine in biological fluids is described. The reaction appears to be specific for isoniazid and *d*-cycloserine. The method is simple, and requires only one reagent, $Na_3[Fe(CN)_5NH_3]$, which can easily be obtained and which is stable over long periods.

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EINE NEUE METHODE ZUR ACETONBESTIMMUNG IN DER ATEMLUFT

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Bis heute sind etwa 15 verschiedene Methoden zum quantitativen oder qualitativen Nachweis von Aceton in der Exspirationsluft angegeben worden. Es sind dabei zu unterscheiden Methoden mit einer feststehenden Versuchsdauer ohne Messung der ausgeatmeten Luftmenge[1–16] und Methoden, bei denen eine abgemessene Luftmenge[1, 2, 6, 17–22] untersucht wird. Für klinische Zwecke eignen sich wenig diejenigen quantitativen Nachweise, die die Gewinnung von Alveolarluft voraussetzen[1, 2, 18, 22]. Bei Bewusstlosigkeit oder Dyspnoe wird man nur die gemischte Exspirationsluft untersuchen können.

Als Fehlermöglichkeit beim Sammeln einer bestimmten Luftmenge kommt in Betracht die Kondensation von Wasserdampf und damit auch von Aceton. Eigene Untersuchungen ergaben nur bei hohen Acetonkonzentrationen (83–125 μg Aceton/l Exspirationsluft), dass im Atembeutel nach Entleerung eine geringe Acetonmenge (bis zu 5%) zurückblieb.

Von den bisher bekannten Methoden, bei denen eine abgemessene Luftmenge[1, 2, 6, 17–22] untersucht wird, sind nach den Angaben in der Literatur einige nicht empfindlich genug[1, 6, 18, 19, 22] oder verhältnismässig zeitraubend[6, 19, 22] oder am Krankenbett nicht immer verwendbar[2, 6, 17, 20–22]. Erwünscht ist daher eine möglichst spezifische, empfindliche, genaue sowie in der Durchführung einfache Methode, die in kurzer Zeit die Bestimmung der Acetonkonzentration in der gemischten Exspirationsluft gestattet.

METHODIK

Prinzip. Eine konstante Luftmenge wird in einen Atembeutel ausgeatmet. Der Atembeutel wird sofort nach der Füllung entleert. Die Luft gelangt in eine salzsaure Lösung von 2,4-dinitrophenylhydrazin. Das in der Luft enthaltene Aceton reagiert mit 2,4-dinitrophenylhydrazin unter Bildung von Aceton-2,4-dinitrophenylhydrazon, das anschliessend mit Tetrachlorkohlenstoff extrahiert wird. Nach Zugabe von Natronlauge und Abpipettieren der wässrigen Schicht wird die Konzentration von Aceton-2,4-dinitrophenylhydrazon elektrophotometrisch bestimmt.

Reagenzien. (1) 0.2%ige Lösung van 2,4-dinitrophenylhydrazin in 2 *N* Salzsäure. Um nennenswerte Extinktionsunterschiede der einzelnen Leerwerte oder gleicher Konzentrationen des Reaktionsproduktes zu vermeiden, kann man die Lösung mit Tetrachlorkohlenstoff schütteln und filtrieren[23]. (2) Tetrachlorkohlenstoff. (3) 5 *N* Natronlauge. (4) Sämtliche Reagenzien p.a.

Atembeutel (Fig. 1): Zum Sammeln einer konstanten Luftmenge dient ein nach eigenen Angaben modifizierter Atembeutel (Hersteller: Draegerwerk, Lübeck) mit

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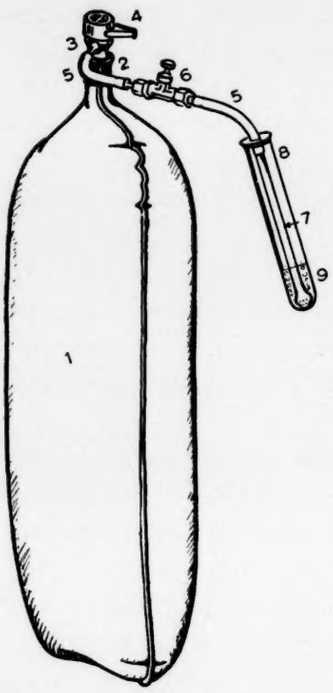
einer dünen Gummieinlage. Das Fassungsvermögen beträgt 24 l. Innerhalb von 10 min tritt kein merklicher Acetonverlust im gefüllten Atembeutel ein. Das tüllenartige Mundstück kann leicht zwischen die Zahnreihen geschoben werden. Das Ventil gestattet nur die Exspiration in den Atembeutel. Da bei der Exspiration kein nennenswerter Widerstand zu überwinden ist, lässt sich der Atembeutel auch bei dyspnoischen und bewusstlosen Patienten anwenden.

Die Gewinnung der gemischten Exspirationsluft soll ohne Hyperventilation erfolgen. Der Atembeutel ist im allgemeinen nach 4–5 min gefüllt: bei einem komatösen Kranken vergingen allerdings 20 min.



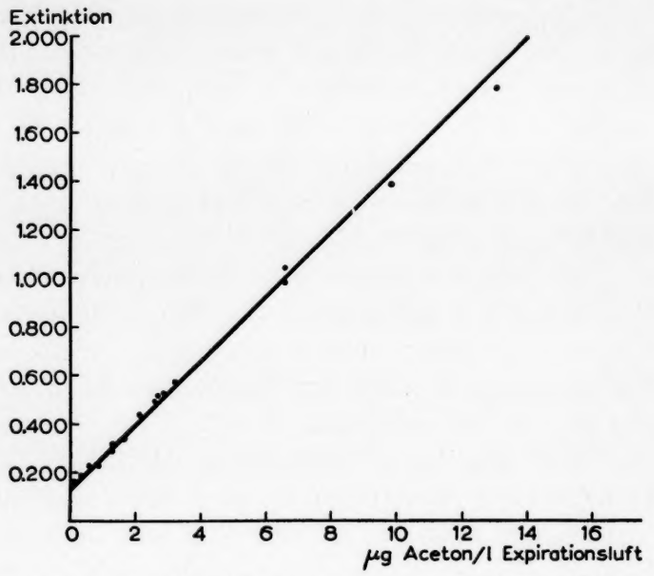


Fig. 2. Acetonbestimmung in der Atemluft. Eichkurve. D = 0.5 cm, Hg 436 mµ, 5 ml 2,4-DNPH.

Fig. 1. Atembeutel. (1) Atembeutel, (2) Ansatz des Atembeutels, (3) Metallgehäuse (Mundstück. Ventil), (4) Mundstück, sowie (5) Zwischenstück aus Gummi, (6) Metallröhre mit Stellschraube, (7) Glasröhre mit Öffnungen für die Luftdurchtritt, (8) Grosses Reagenzglas, (9) Reagenz.

Das Einleiten der Exspirationsluft in das Reagenz wird unter geringem Druck sofort nach der prallen Füllung des Atembeutels vorgenommen, um eine Kondensation von Wasserdampf und damit auch von Aceton möglichst niedrig zu halten. Nach Öffnen der Stellschraube in der Metallröhre (Fig. 2) gelangt die Luft durch eine Glasröhre in ein grosses Reagenzglas mit 5 ml 2,4-dinitrophenylhydrazin in salzsaurer Lösung. Die Glasröhre hat an ihrem freien Ende mehrere kleine Öffnungen, damit eine ausreichende Durchmischung der acetonhaltigen Exspirationsluft mit dem Reagenz gewährleistet ist. Die Entleerung soll in etwa 4 min und möglichst gleichmässig erfolgen. Nach Verschliessen des Reagenzglases mit einem Glasschliffstopfen wird etwa 20 sec lang kräftig geschüttelt. Die Lösung bleibt 5 min stehen [24]. Je nach der Konzentration des entstandenen Aceton-2,4-dinitrophenylhydrazons färbt sich die Lösung gelbgrün bis goldgelb, oder es treten kleinflockige Niederschläge im Lumen und an der Wand der Glasröhre und des Reagenzglases auf.

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Die Extraktion mit Tetrachlorkohlenstoff und Alkalisierung. 5 ml Tetrachlorkohlenstoff werden zum Inhalt des Reagenzglases gegeben. Nach etwa 20 sec langem Schütteln zeigt die Tetrachlorkohlenstoffschicht bei mässig oder stark erhöhten Acetonkonzentrationen einen gelbgrünen bis goldgelben Farbton, der von dem quantitativ extrahierten Aceton-2,4-dinitrophenylhydrazon herrührt. Flockige Niederschläge bei sehr hohen Konzentrationen des Reaktionsproduktes lösen sich schnell in Tetrachlorkohlenstoff*. Nach einigen Minuten wird zur Verstärkung der Farbintensität[17] 1 ml 5 *N* Natronlauge hinzugefügt. Anschliessend wird 20 sec lang kräftig geschüttelt. Nach 5 min wird die obere, wässrige Schicht abpipettiert und verworfen.

Die elektrophotometrische Bestimmung erfolgt im Elektrophotometer "Eppendorf" (Hersteller: Firma Netheler und Hinz, Hamburg) bei 436 mμ; die Schichtdicke der Küvette beträgt 0.5 cm. Etwa 10 min nach Abpipettieren der wässrigen Schicht wird die jetzt fast ausschliesslich aus Tetrachlorkohlenstoff mit extrahiertem Aceton-2,4-dinitrophenylhydrazon bestehende Lösung in die Küvette gefüllt. Bei raschem Eingiessen können Reste der wässrigen Schicht in die Küvette gelangen und die elektrophotometrische Bestimmung empfindlich stören. Es wird gegen Tetrachlorkohlenstoff abgelesen. Da Tetrachlorkohlenstoff in sehr geringem Masse auch 2,4-dinitrophenylhydrazin in salzsaurer Lösung extrahiert und daher eine Leerabsorption vorhanden ist, empfiehlt es sich, bei jeder Bestimmungsreihe einen Leerwert mitlaufen zu lassen. Die Extinktion des Leerwertes liegt im Mittel bei 0.120. Bei längerem oder kürzerem Warten nach Abpipettieren der wässrigen Schicht ändern sich die Extinktionen der Ansätze mit dem Reaktionsprodukt und der Leerwerte in nahezu gleichem Masse. Fig. 2 zeigt die Eichkurve. Das Lambert-Beer'sche Gesetz wird bis zu einer Konzentration von 12.50 μg Aceton/l Exspirationsluft erfüllt. Die Fehlerbreite beträgt 4%, die Empfindlichkeit 0.063 μg Aceton/l Exspirationsluft (1.5 μg Aceton/5 ml Reagenz).

Als *Nüchternwerte* wurden bei gemischter Kost bis zu 0.20 μg Aceton/l Exspirationsluft gefunden.

Bei über 150 Bestimmungen lagen die Werte zwischen 0.063 und 143.8 μg Aceton/l Exspirationsluft.

DISKUSSION

Die Methode wurde für klinische Zwecke zur Orientierung über die Schwere einer Ketose ausgearbeitet. In etwa 30 min lässt sich die Acetonkonzentration in der gemischten Exspirationsluft mit verhältnismässig grosser Spezifität, Empfindlichkeit und Genauigkeit sowie Einfachheit in der Durchführung bestimmen.

Die geringfügige Kondensation an Aceton bei der Abkühlung der Exspirationsluft bis auf etwa Zimmertemperatur (im Atembeutel mehrere Minuten nach der Füllung) wurde nicht berücksichtigt. Ebenso wurde der Einfluss von Temperaturänderungen vernachlässigt; das Volumen der Exspirationsluft vermindert sich bei Abkühlung von etwa 31° in der Mundhöhle auf 20° im Atembeutel um etwa 4%.

Bei eigenen Versuchen fand sich kein Unterschied zwischen der Acetonkonzentration in der Alveolarluft und der in der gemischen Exspirationsluft. Es liess sich nicht entscheiden, ob tatsächlich in beiden Fällen die Acetonkonzentration gleich war oder

* Eine Verdünnung kann ohne Beeinträchtigung der Reaktion mit Tetrachlorkohlenstoff erfolgen.

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nicht doch eine geringe Differenz vorlag, für deren Erfassung die Empfindlichkeit der Methode nicht mehr ausreichte.

Die Fehlerbreite beträgt etwa 6%, davon 4% bei der elektrophotometrischen Bestimmung.

Die Reaktion ist verhältnismässig spezifisch. Da 2,4-dinitrophenylhydrazin mit Carbonylgruppen reagiert, werden auch Acetaldehyd, Paraldehyd und Formaldehyd erfasst. Die Acetaldehydkonzentration in der Exspirationsluft ist erhöht nach Alkoholgenuss[25], besonders bei Antabusbehandlung[25] oder nach Dipyrin[26]. Nach Gabe von Paraldehyd in Form einer 10%igen Lösung (Schlafmittel) tritt eine deutliche oder starke Reaktion auf. Bei Gesunden war keine Formaldehydausscheidung nachweisbar. Erst hohe Formaldehydkonzentrationen verfälschten die Ergebnisse.

Die verhältnismässig grosse Luftmenge (24 l) erlaubt die Bestimmung auch kleiner Acetonkonzentrationen.

ZUSAMMENFASSUNG

Für klinische orientierende Untersuchungen wird eine neue Methode zur Acetonbestimmung in gemischter Exspirationsluft angegeben. Eine konstante Luftmenge (24 l) wird in einen für Aceton fast undurchlässigen, wenig elastischen und mit einem Ventil versehenen Atembeutel ohne nennenswerten Widerstand ausgeatmet. Sofort nach Füllung des Atembeutels wird die Luft in eine salzsaure Lösung von 2,4-dinitrophenylhydrazin geleitet. Aceton reagiert mit 2,4-dinitrophenylhydrazin unter Bildung von Aceton-2,4-dinitrophenylhydrazon. Anschliessend wird mit Tetrachlorkohlenstoff das Reaktionsprodukt extrahiert. Nach Alkalisierung und Abpipettieren der wässrigen Schicht folgt die elektrophotometrische Bestimmung des in der Tetrachlorkohlenstoffschicht enthaltenen Aceton-2,4-dinitrophenylhydrazons bei 436 mμ.

SUMMARY

A NEW METHOD FOR DETERMINING ACETONE IN EXPIRED AIR

For clinical orientation tests a new method for determining acetone in mixed expired air was developed. A constant quantity of air (24 l) is expired into an airbag equipped with a valve, which presents very little resistance to the entrance of the air. This bag is not very elastic and is practically impermeable to acetone. Immediately after the bag is filled the air is led into a solution of 2,4-dinitrophenylhydrazine in hydrochloric acid. Acetone reacts with the 2,4-dinitrophenylhydrazine to give the 2,4-dinitrophenylhydrazone. The reaction product is then extracted with carbon tetrachloride. After alkalinization and removal of the aqueous layer with a pipette, the acetone 2,4-dinitrophenylhydrazone in the carbon tetrachloride layer is determined electrophotometrically at 436 mμ.

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DETERMINATION OF BARBITURATES BY AUTOMATIC DIFFERENTIAL SPECTROPHOTOMETRY*

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The quantitative determination of barbiturates by virtue of their characteristic spectra in the ultraviolet portion of the spectrum has been of considerable value[1-6]. The absorbance differences obtained by subtraction of the pH-10.5 spectrum from the spectrum obtained in much stronger alkali (0.45 *N* NaOH) show characteristics which are useful for both qualitative and quantitative determinations of barbiturates present in physiologic media under toxicological conditions[6, 7]. Analytical pharmaceutical applications are also of considerable importance[8].

There is difficulty in manually obtaining a pair of spectra for each sample which is to be subjected to quantitation. This is true even though the ultraviolet spectra represent relatively crude graphs with absorbances taken at a limited number of wavelengths as a time expediency measure[10]. The use of the differential characteristics of an automatic recording spectrophotometer obviates the time factor involved in the manual recording of spectra required for individual sample analysis. It precludes the use of blanks since the two aliquots of barbiturate blank each other just as appropriately as if the two samples were analyzed individually. This is aided by virtue of the fact that the sodium hydroxide and sodium hydroxide–ammonium chloride solutions used as the two solvents for barbiturate yield zero absorbance against each other over the range of 340 mμ to 230 mμ. It eliminates the subtraction of one spectrum from the other spectrum for each sample, since the technic of instrumentation automatically presents the subtractive effect.

The present investigation describes the use of DK-2 automatic ratio-recording spectrophotometer for a simplified modification of the differential spectra technic for barbiturates. Automatic recording of the composite spectrum of a barbiturate is more rapidly accomplished than in present procedures and makes routine graphing of many spectra possible in a laboratory involved with the determination of barbiturates in a large number of samples of blood, urine, tissue homogenates, or bath water from hemodialysis[11].

METHOD

Reagents

Chloroform. Spectranalyzed reagent, or redistilled analytical reagent.

Sodium hydroxide, 0.45 *N*.

Ammonium chloride, 16% for macro determination and 20% for the micro determination.

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Apparatus

Beckman, DK-2 automatic ratio-recording spectrophotometer. Silica cuvettes, macro and micro, 1-cm light paths.

Procedure

Instrumentation. Zero the instrument utilizing the hydrogen lamp source and the photomultiplier tube at IX using the following conditions:

Wavelength	340 mμ
Approximate slit width	0.04 mm
Sensitivity	100
Time	2 min
Time constant	0.2
Initial range	0–100
Scale, initial	Per cent T
Final range	—0.3 to +0.7
Scale, final	absorbance

Zeroing the instrument can be accomplished by first putting 0.45 *N* sodium hydroxide solution in either cuvette and the sodium hydroxide–ammonium chloride solution in the other cuvette. In actual practice, however, zeroing against air has achieved similar results. After zeroing the instrument, change the range to −0.3 to +0.7 and the scale to absorbance with the cuvettes in the cuvette holder. The pen will now automatically move to 0.3 absorbance on the chart paper. Adjust the pen to 0.5 on the chart at 340 mμ with the 100% control and this now becomes the zero absorbance for both negative and positive spectra. Record the differential spectrum from 340 mμ to 220 mμ using the previously specified conditions.

Analysis of unknown solutions (macro)

Pipet 1–5 ml of serum or blood (1–5 g tissue homogenate) and 50 ml of redistilled or spectral grade chloroform into a separatory funnel and extract for a period of 5 min. A pH 7.4 buffer can be added although in most instances, it has been found to be unnecessary. Filter the chloroform layer into a clean dry separatory funnel, take a 40–45 ml aliquot, add 7 ml of 0.45 *N* NaoH and back-extract into the aqueous layer with a second 5-min shaking period. In routine work, it has been found that using the entire chloroform extract achieves similar results and has been found to be adequate for this purpose. Discard the chloroform and centrifuge the alkali phase. Remove the small amount of chloroform using a syringe and a long needle. This obviates the problem of bubble formation when pipetting off aliquots of the alkaline extract over the chloroform remaining after centrifugation. Pipet 3 ml of the alkaline extract into two silica cells. To one add 0.5 ml of the 0.45 *N* NaOH and to the other add 0.5 ml of 16% NH_4Cl. Read the differential spectrum of the well mixed solutions in the range of 340 mμ to 220 mμ using the previously described conditions.

Analysis of unknown solutions (micro)

Pipet 0.1–0.5 ml of the solution to be analyzed into 10 ml of redistilled or spectro-analyzed chloroform and extract for a period of 5 min. Centrifuge the mixture and then carefully aspirate off the upper layer. Pipet 1.0 ml of 0.45 *N* NaOH into a 9-ml aliquot of the chloroform solution and re-extract the barbiturate for 5 min. Centrifuge and carefully remove the lower layer using a long needle on a syringe. Pipet 0.4-ml

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aliquots of the alkaline extract into two micro cuvettes and then add 0.05 ml of 20% NH_4Cl to one cuvette and 0.05 ml of 0.45 *N* NaOH to the other cuvette. 20% ammonium chloride is used in the micro procedure to maintain the same sodium hydroxide–ammonium chloride concentration ratio as in the macro procedure. Read the differential spectrum of the well mixed solutions in the range of 340 mμ to 220 mμ using the conditions previously described, and the smallest masking slit of the DK-2 filter holder turned parallel to the instrument slit.

DISCUSSION

In order to justify the use of the automatic differential spectrum as a substitute for the subtraction of the two individual spectra of a barbiturate at two different alkalinities, the following experiment was performed. The spectra of two aliquots of phenobarbital, 20 mg/ml of 0.45 *N* NaOH were graphed in normal fashion against their appropriate blanks where one sample was acidified to pH 10.5 with 16% NH_4Cl.

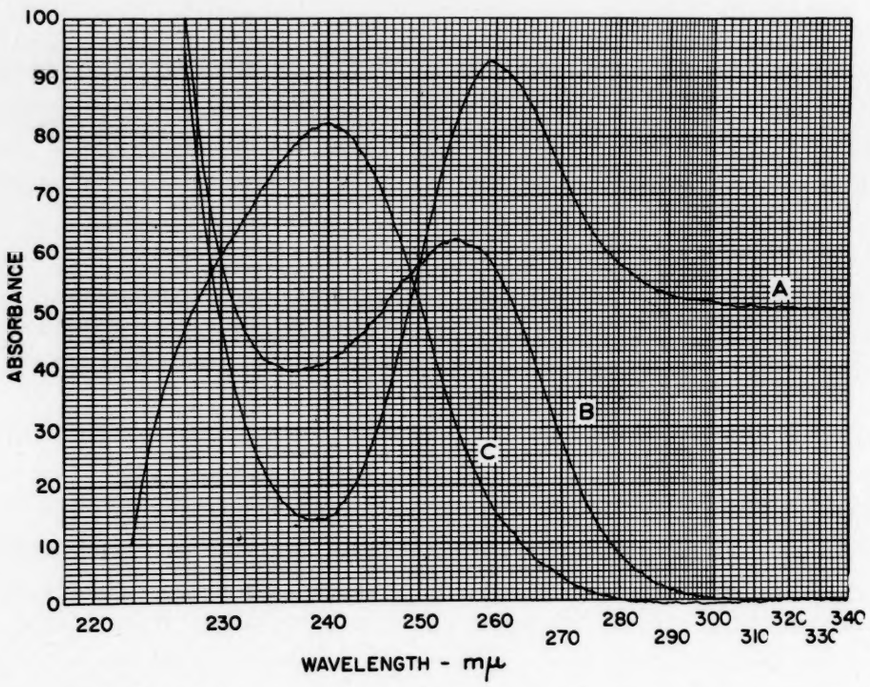

Fig. 1. Curve B, normal spectrum (very alkaline), Curve C, normal spectrum (pH 10.5), and Curve A, automatic composite differential spectrum.

These are shown as curve B (very alkaline) and curve C (pH 10.5) of Fig. 1. When the two samples were graphed differentially against each other, curve A was obtained. The integral nature of this composite hypsochromic spectrum includes internal blanking and an automatic subtraction of two spectra for the observed effect. The absorbance differences of the two curves, C and B, are similar to the differential spectrum shown as curve A.

The measurements of the differential characteristics of a variety of barbiturates are graphically illustrated in Fig. 2 and Fig. 3. The zero line for obtaining the beginning of the differences in the two spectra, pH 10.5 and 0.45 *N* NaOH are shifted up and down from zero to better delineate the different species. The minima and maxima can be alternated by the simple expedient of switching sample cell to reference cell.

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Either cell, therefore, can exhibit the minimum or maximum absorptivity depending on whether the cell is arbitrarily put in the reference or the sample side. The isosbestic points obtained by not using the differential spectra correspond to the intersections of the differential spectra and their zero line. The different species show different isosbestic points.

A number of concentrations of phenobarbital were graphically scanned using the differential technic and the results are shown in Fig. 4. Excellent linear obeyment of Beer's law is indicated.

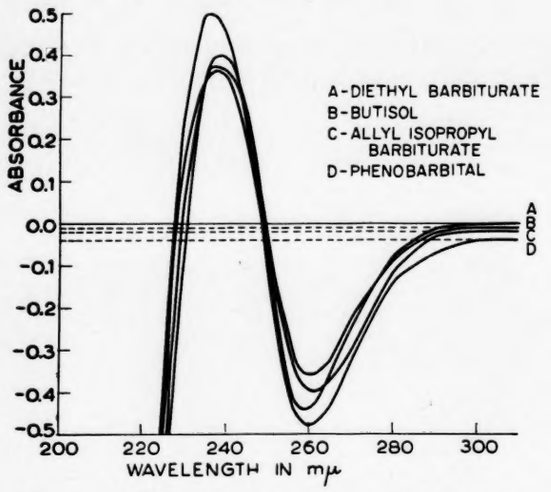

Fig. 2. Differential absorption spectra of several barbiturates.

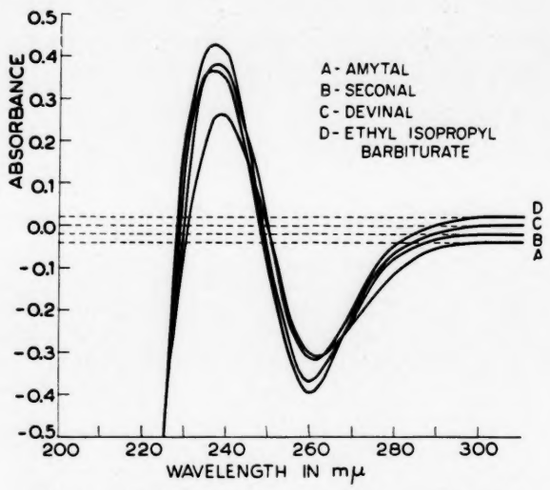

Fig. 3. Differential absorption spectra of several barbiturates.

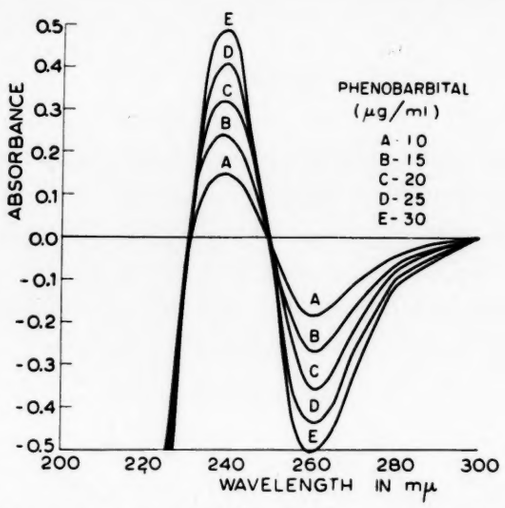

Fig. 4. Differential absorption spectra of various concentrations of phenobarbital.

A differential spectra was obtained with a solution of barbiturate in both a standard cuvette and in micro cuvettes whose dimensions were 50 × 3 × 10 mm. The only difference in technic involved the use of a DK-2 masking slit approximately 1 × 8 mm in dimensions, turned parallel to the instrument slit instead of leaving it in its normal perpendicular position. Since the spectra obtained in both cases were the same, one can deal with smaller amounts of blood and preclude the use of a micro diaphragm attachment for this purpose. These masking slits are much handier than the diaphragms commercially available and fit in the filter carrier of both the

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manual and automatic instruments. Smaller slits than those which are in the DK-2 can easily be made.

Recovery studies were carried out in which varying amounts of several barbiturates were added to different bloods which were determined to be barbiturate-free. Since barbiturates are not blood constituents recoveries could only be ascertained by adding absolute amounts of barbiturates to blood in an effort to see if the determinative technic is capable of accurate quantitation. As far as is known to us, no other procedure for recovery from human blood is available. Every recovery was made from a different blood and in no circumstance was a recovery made from a previously used blood. These recoveries are illustrated in Table I and it can be seen that the amounts found are excellent indicators of the absolute quantities known to be present.

TABLE I

RECOVERY OF SEVERAL BARBITURATES FROM BLOOD

Sample (5 ml)	*Barbiturate*	*mg present*	*mg found*
Blood A	Sodium Seconal	138	136
B	Sodium Seconal	110	116
C	Sodium Seconal	83	84
D	Sodium Amytal	155	151
E	Sodium Amytal	109	107
F	Sodium Amytal	78	77
G	Phenobarbital	156	155
H	Phenobarbital	109	106
I	Phenobarbital	94	87

SUMMARY

An investigation was carried out on the determination of barbiturates in the ultraviolet portion of the spectrum using the differential characteristics of an automatic recording spectrophometer. Recovery studies were carried out on absolute quantities of various barbiturates prior to subjecting them to chemical manipulation as well as to a group of randomly selected bloods which were shown to be barbiturate-free. Representative differential spectra of a series of barbiturates are described. The differential procedure was also adapted to micro analysis.

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STUDIES IN REFRACTORY PERNICIOUS ANAEMIA
AN INVESTIGATION WITH RADIOACTIVE VITAMIN B_{12}-^{58}Co

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INTRODUCTION

With the discovery of Vitamin B_{12}, the anti-pernicious anaemia factor, the pernicious character of this blood disease has been virtually constrained.

It has since become evident, that the therapy could be improved by combining the extrinsic factor (Vit. B_{12}) with a highly purified pylorus extract (intrinsic factor; ferment of CASTLE[1]) in oral administration—thus promoting the resorption in the gastro-intestinal tract.

The possibility of avoiding the ever-repeated intramuscular injections of liver extracts must undoubtedly be regarded as an important improvement, not only from the point of view of therapy, but especially because of a better understanding of the physiological chemical aspects of this disease.

Many problems still arise, *e.g.* the observation by KILLANDER[2] that oral maintenance therapy of pernicious anaemia patients with cyanocobalamin and hog intrinsic factor concentrates may lead to reduced absorption of Vit. B_{12} in a number of patients. This has been confirmed by several other investigators[3–10].

As the cause of the development of this refractoriness is not yet understood, we have studied the influence of sterilisation of the intestinal tract on the absorption of radioactive Vit. B_{12} in 2 refractory patients, using a modified SCHILLING[11] technique with ^{58}Co-labeled cyanocobalamin. Furthermore, we have investigated whether the absorption of Vit. B_{12} could be improved in one refractory patient, by daily oral administration of dried liver as the source of Vit. B_{12} in combination with various types of hog intrinsic factor concentrates using the serum-B_{12} levels as the criterion for improved B_{12}-absorption.

Although only three, refractory patients were available for our studies, we present the results in this article, for we do not expect to be able to extend our investigations.

MATERIAL AND METHODS

(1) *Patients*

15 pernicious anaemia patients, under observation from 6–36 months, were given oral treatment of 1–2 tablets Bifacton per day during the whole period of observation.

The original diagnosis had been based in all cases on the following criteria: Hyperchromic anaemia (colour index > 1.3) and an enlarged diameter. Megaloblasts in the red bone-marrow. Absence of "free" HCl in the gastric juice, even after the injection of histamin.

A slightly increased pH-resistance[12, 13] was found, and in some cases the life-span of the erythrocytes was estimated with radioactive $Na_2{}^{51}CrO_4$. At a normal

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apparent half-survival time of $t^{1/2}_A = 30 \pm 3$ days, we found in these cases of pernicious anaemia: $t^{1/2}_A = 20$–26 days.

The case histories of the three patients reported on in our present study are given under RESULTS. See also Table II.

(2) *Intrinsic factor preparations and composition of tablets*

The Bifacton tablets used for the maintenance therapy were the commercial preparations*. They contained 0.5 U.S.P.-unit (oral) of Vit. B_{12} and intrinsic factor concentrate per tablet, *i.e.* 7.5 μg cyanocobalamine + 15–25 mg intrinsic factor concentrate, prepared from hog pyloric mucosa. B.F.S. 35, used for the urinary excretion test (see point (4) below), and B.F.S. 209, used for the preparation of experimental tablets (see Table III), were two samples of intrinsic factor concentrate, such as is used for the preparation of Bifacton tablets; 30 mg of B.F.S. 35, or 41.5 mg of B.F.S. 209, after combination with 15 μg B_{12}, would give 1 U.S.P. unit (oral).

I.F.–2190, also given in experimentally tablets, was lyophilized and defatted hog pyloric mucosa, obtained in a yield of 170 mg/g of fresh starting material. For the preparation of the test tablets, dried and defatted liver powder with a Vit. B_{12}-activity of 0.0031 μg/mg was used throughout; for exact composition see Table III.

(3) *Urine excretion test (U.E.T.) without intrinsic factor (I.F.)*

In our investigation this test was carried out as follows: The fasting patient was given orally: 0.5 μg Vit. B_{12}–^{58}Co**.

Directly afterwards 1 mg of non-radioactive Vit. B_{12} was injected intramuscularly. The 24-h urine was collected, and the amount of radioactivity excreted, determined. After 24-h a second intramuscular injection with 1 mg Vit. B_{12} was given, the urine was collected for another 24 h, and the radioactivity measured. (see point (5) below).

(4) *Urine excretion test (U.E.T.) with intrinsic factor (I.F.)*

As a standard intrinsic factor, in this test we used a preparation B.F.S. 35; 30 mg hereof combined with 15 μg Vit. B_{12}, is equivalent to 1 U.S.P. unit (oral). The dose chosen for this U.E.T. was 12 mg B.F.S. 35, equivalent to 0.4 U.S.P. unit.

The patient received orally 0.5 μg Vit. B_{12}–^{58}Co, homogenized with 12 mg intrinsic factor (B.F.S. 35).

The continuation of this test was, as described in point (3) above. The patients were not allowed any Vit. B_{12} therapy 3 days before the start of this test; 2 h after its beginning they were allowed to eat, with the exception of meat and fish.

Intervals of at least 7 days were allowed between U.E.T.'s in the same patient.

(5) *Radioactive measurement*

The determinations of ^{58}Co were performed with a well-type scintillation counter, a NaI crystal, activated with thallium (4 π counting). The Superscaler (Tracer-Lab.) was used for counting. With a screening of 5 cm lead the background varied from 600–700 counts/min.

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* N.V. Organon, Oss, The Netherlands.

** Activity at time zero: 3.37 μC/μg ^{58}Co has a half-life of 72 days; therefore it is more fitted for diagnostic purposes then ^{60}Co ($t^{1/2} = 5.27$ y). ^{58}Co has a γ-radiation of 0.81 MeV ($\sim$ 100%); $\beta^+ = 0.47$ MeV (*ca.* 15%) with E.C. (*ca.* 85%).

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The urine was measured in graduated tubes, containing 4 ml of the test solution. Depending on the activity, the counting time varied from 30–120 min, in order to obtain a total count of at least 20,000.

To prevent the absorption of radioactive Vit. B_{12}–^{58}Co on the glass walls of the urine-collecting bottles, a solution of non-radioactive Vit. B_{12} was added as a carrier, before collecting the urine. The maximum concentration of ^{58}Co excreted in the urine, amounted to only 0.3 μC/l in these tests, but in most cases it was 20–30 times less.

Nevertheless it was frequently possible to count the urines, without concentrating them, even when the excreted amounts of ^{58}Co were very small. This was repeatedly checked by concentrating the urine 3–30 fold. Two examples are given in Table I.

TABLE I

REPRODUCIBILITY OF ^{58}Co-DETERMINATIONS IN URINE, WITH AND WITHOUT CONCENTRATION

	Background 30 min	*4 ml urine 30 min*	*Δ in counts/min*	*Per cent of administered amount of ^{58}Co in 0–24-h urine*
Normal urine	19,536	23,059	116	14
Concentrated urine 300 to 38 ml	19,375	45,466	110	13
Normal urine	20,865	23,251	79	4
Concentrated urine 300 to 108 ml	20,865	27,552	80	4

The very good reproducibility, with and without concentration is clearly demonstrated, bearing in mind that the deviation of a sufficiently high count is proportional to its square root.

(6) *Vitamin B_{12} in blood serum*

The Vit. B_{12}-level in the blood serum was assayed microbiologically by Ir. P. A. VAN HEMERT (Microbiological Department, N.V. Organon, Oss, The Netherlands), using the slightly modified U.S.P. XV method with *Lactobacillus leichmannii*.[14]

RESULTS

(1) In 12 out of 15 patients with pernicious anaemia on oral maintenance treatment with Bifacton, the U.E.T. (without I.F.) gave the expected results, *viz.* a very low percentage of radioactivity recovered in total 48-h urine after the oral administration of radio-B_{12} alone (0–4%). A marked increase in excretion (6–20%) resulted,

TABLE II

AVERAGES OF SOME HAEMATOLOGICAL DATA, AS FOUND IN 12 PATIENTS WITH PERNICIOUS ANAEMIA recorded at the time of relapse and after 6–36 months of oral treatment with 1–2 tablets of bifacton per day

	Red corpuscles million/μ l	*Hb g%*	*Colour index*	*Diam. μ*	*White corpuscles per μl*	*Reticulocytes %*	*Max. day*
Before treatment	1.3	6.2	1.5	7.9	3800	15.0	6–8
After treatment	4.5	14.3	1.0	7.3	6700		

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when the radio-B_{12} was given together with 12 mg of B.F.S. 35. These values approached the level found in normals with radio-B_{12} alone (6.5–30%).

These results were completely in agreement with the clinical status of the patients, who were in excellent condition and had normal haematological values.

In Table II, the averages of some haematological data of these 12 patients are given, both at the time of relapse (before treatment) and at the time just before the SCHILLING-tests were performed (after oral treatment).

(2) In two other patients (A and B) the results of the U.E.T. showed that the patients were refractory to the intrinsic factor (see Table III), as the U.E.T. *with intrinsic factor* did *not* give any marked rise in the excretion of radioactivity, as compared to the test results with radio-B_{12} alone. Therefore these patients were used in our first investigation concerning the influence of sterilisation of the intestinal tract on the absorption of radio-B_{12} + intrinsic factor. The case histories of these patients are given below.

Patient A.

At first hospitalisation the laboratory investigations supplied the following results:
Red corp., $1.1 \cdot 10^6$/mm³; Hb, 6.0 g %; D, 7.8 μ; white corp., 4000/mm³.
Diff: segm. 71%, lymph. 29%; strong anisocytosis, and hypersegmentation of the granulocytes. The bone-marrow showed many megaloblasts and giant-granulocytes.

A histamine-refractory achylia gastrica was demonstrated. The initial pH-haemolysis[12, 13] amounted to pH = 5.5, while the Vit. B_{12}-concentration in serum was estimated twice on two successive days, and revealed 37 $\mu\mu$g/ml and 30 $\mu\mu$g/ml respectively. The U.E.T. without I.F. confirmed the diagnosis: pernicious anaemia. No aberrations were found in the gastro-intestinal tract and the kidney functions proved to be normal.

From the anamnesis it appeared, that the patient, prior to admission had used Binaemon tablets, and during the last three weeks Bifacton to the absurd amount of 9 a day.

Patient B.

In this case pernicious anaemia had been diagnosed, about two years earlier. All symptoms, mentioned in point (1) under MATERIALS AND METHODS had been found. At that time the patient had reacted very well to the oral therapy (Vit. B_{12} + intrinsic factor), *e.g.* after seven days the reticulocytes increased to 30%. The blood picture became normochrome with a Hb-percentage of 15.8 g %. All complaints disappeared and an oral maintenance therapy of 2 tablets Bifacton per day was prescribed which the patient followed up to his readmission. In the framework of this research the patient was readmitted to hospital, with the following diagnosis red corp., $3.8 \cdot 10^6$/mm³; Hb, 14.0 g%; D, 7.6 μ, white corp., 5200/mm³. The erythrocytes showed a clear anisocytosis; the granulocytes were hypersegmented. The initial pH-haemolysis amounted to pH 5.3; the Vit. B_{12} content of the serum was 60 $\mu\mu$g/ml.

TABLE III

RESULTS OF URINARY EXCRETION-TEST IN 2 REFRACTORY PATIENTS A AND B BEFORE AND AFTER STERILISATION OF THE INTESTINAL TRACT WITH NEOMYCIN

Oral dose during U.E.T.	*Percent radioactivity**			
	Patient A		*Patient B*	
	24-h urine	*48-h urine*	*24-h urine*	*48-h urine*
Before sterilisation of the intestinal tract with Neomysin				
0.5 μg radio-B_{12} alone	0.7	1.0	0.7	1.0
0.5 μg radio-B_{12} + 12 mg B.F.S. 35	0.9	1.1	2.3	3.5
After sterilisation of gastro-intestinal tract with Neomycin				
0.5 μg radio-B_{12} + 12 mg B.F.S. 35	0.7	0.9	1.0	1.5
0.5 μg radio-B_{12} + 100 ml normal, neutr. gastric juice	13.8	18.8	–	–
0.5 μg radio-B_{12} + 12 mg B.F.S. 35	0.8	1.1	–	–

* Radioactivity in urine, expressed in percentage of radioactivity given as an oral dose.

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The sterilisation of the gastro-intestinal tract in these two patients was achieved by daily oral administration of 4 × 500 mg Neomycin, for a period of one week. After this treatment some more U.E.T.'s were performed, as summarized in Table III.

After sterilization of the gastro-intestinal tract, no improvement of the resorption could be demonstrated. Attention should be paid to the fact, that when the U.E.T. was repeated with 100 ml normal, neutralised human gastric juice resorption was excellent, as was manifested in an urine excretion of ^{58}Co up to 18.8%.

(3) The case history of the third patient (C) seemed to indicate, that here too, refractoriness had developed.

Patient C.

This patient was first seen in 1957 in a condition of relapse. The diagnosis: pernicious anaemia was established as described in point (1) under MATERIAL AND METHODS (Red corp., 1.7 million/μl; Hb, 7.0 g%). The response to oral therapy, 2 tablets of Bifacton per day, was quite satisfactory.

A reticulocyte maximum of 24.0% was found on the 6th day, and the increase in red corpuscles in the first 21 days of treatment was 1.7 million/μl. During the same period the serum B_{12}-level increased from 20 to 134 $\mu\mu$g/ml. After this initial treatment the patient was discharged, and oral therapy was continued with 2 tablets Bifacton per day.

Six months later, the serum B_{12}-level was found to have dropped again below the borderline of 100 $\mu\mu$g/ml; values as low as 60 and 50 $\mu\mu$g/ml were found in two serum samples. The blood again showed anisocytosis and hypersegmentation; the average diameter of the erythrocytes was 7.7 μ.

In case this patient had become truly refractory after this short period of six months of oral treatment, it seemed interesting to study the changes in the serum B_{12}-level, after oral treatment with tablets of various compositions, differing from the normal Bifacton.

A check on the reduced absorption of radio-B_{12} in the U.E.T. with I.F., at this stage, was not advisable, because the massive doses of non-radioactive Vit. B_{12} would increase the body stores of Vit. B_{12} to such an extent that the serum B_{12}-level could *not* be used for demonstrating improved absorption of Vit. B_{12} under the influence of oral treatment with the tablets to be tested. Therefore patient C's oral treatment was changed from Bifacton to other tablets, and the serum B_{12}-level was determined at intervals of 3 weeks. All tablets tested (for composition see MATERIAL AND METHODS, point 2) contained dried and defatted liver powder as the source of Vit. B_{12}, instead of cyanocobalamin. The composition of the various oral doses and the results of this investigation are summarized in Table IV.

TABLE IV

Tablets administered		*R.P. 1693*	*R.P. 1884*	*R.P. 1891*
I.F. in preparation		*B.F.S. 209*	*B.F.S. 209*	*I.F. 2190*
Daily dose	*Amount (mg)*	*41.4*	*13.8*	*124.2*
	Equivalence	*1 U.S.P. unit*	*1/3 U.S.P. unit*	*0.73 g fresh hog pyl. mucosa*
Serum B_{12}-level in $\mu\mu$g/ml after:	3 weeks	60	65	50
	6 weeks	69	50	50
	9 weeks	40	47	45
	12 weeks	47		
	16 weeks	55		
		change to R.P. 1884	change to R.P. 1891	change to U.E.T. (see text)

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After 9 weeks of treatment with R.P. 1891 tablets the condition of the patient did not allow continuation of the oral therapy. Therefore an U.E.T. with 12 mg B.F.S. 35 was performed.

It was found, that the amount of radioactivity excreted in 48 h urine was only 3.5% of that present in 0.5 μg of Vit B_{12}–^{58}Co. This result demonstrated that our patient C was truly refractory.

DISCUSSION

In the refractory pernicious anaemia of these three patients, it appears likely that the failure of the oral therapy with Vit. B_{12} + intrinsic factor must be attributed to the *hog*-intrinsic factor (see Table III). The cause of this phenomenon can be sought in different directions.

(1) TERNBERG AND EAKIN[15] found that normal human gastric juice contains a non-dialysable, heat-labile substance (apoerythein) which combines with Vit. B_{12}, giving a complex in which the vitamin is no longer available for the growth of certain micro-organisms, like *E. coli*, *L. leichmannii* etc. Since the "binding capicity" of the gastric juice of pernicious anaemia patients is low in comparison to that of normal persons, and preparations from hog gastric mucosa (known to contain intrinsic factor activity) contain apoerythein, whereas some of the proporties of the unknown binding substance seem to be comparable to those of to the intrinsic factor, they concluded that apoerythein was probably identical with CASTLE's intrinsic factor.

Although no clinical evidence was given by TERNBERG AND EAKIN, their theory seems attractive, as it offers a new intrepretation for the mode of action of the intrinsic factor.

If the cobalamins in food were bound by the intrinsic factor (resp. apoerythein), they would not be available for the bacteria in the intestines. The intrinsic factor would then protect Vit. B_{12} against the microbial flora and convey it safely to that part of the intestine where it would be absorbed by the mucosa.

One of the theories that can be offered to explain the occurrence of refractoriness after prolonged oral treatment of pernicious anaemia patients with Vit. B_{12} and hog intrinsic factor concentrates, is based on the possible changes in the microbial flora of these patients. It is possible that some of these micro-organisms either inactivate the intrinsic factor or cyanocobalamin, or that they consume the bound B_{12} present in these oral preparations (for review article on binding substances in intrinsic factor preparations see[16]), thereby making the Vit. B_{12} unavailable for the host. Since many refractory patients are able to absorb Vit. B_{12} when *human gastric juice* * is used as the source of the intrinsic factor (see Table III), one would have to assume that these micro-organisms are not able to influence the absorption in the way described above, when human gastric juice is the source of intrinsic factor and/or binding substances. This would not necessarily mean that the *intrinsic factor* is species-specific, for it may be that only the binding substances show some species-specificity.

Our results in patients A and B, summarized in Table III, do not support this theory, for no signs of improved absorption of Vit. B_{12} were seen after sterilization of the intestinal tract with Neomycin.

* A complicating factor in this respect is the observation of GLASS *et al.*[17] that the absorption of B_{12} in a p.a. patient, which had been satisfactory after the use of 100 ml of normal human gastric juice + radio-B_{12}, diminished considerably when the test dose of radio-B_{12} was administered in 190 ml of the same juice.

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Similar observations were made by DR. J. F. ADAMS (Western Infirmary, Glasgow) on a group of refractory patients, using Aureomycin for sterilization.

(2) Recent observations have shown that not only purified hog intrinsic factor concentrates[2, 5, 7–10], but also crude concentrates in the form of desiccated and defatted hog pyloric mucosa[3, 4, 6] can give rise to the development of refractoriness. These results were quite unexpected because the initial response in pernicious anaemia patients in relapse, used as a criterion for the activity of oral preparations, was always satisfactory, and it has never been indicated in the earlier literature that refractoriness could develop after long-term treatment of pernicious anaemia with the oral preparations then available (see[9] for references and discussion).

Since all recent preparations contain cyanocobalamin as the source of Vit. B_{12}, whereas earlier preparations were combinations of hog stomach and dried liver, it seemed interesting to ascertain whether combinations, containing dried liver instead of cyanocobalamin would give a better absorption of Vit. B_{12} in refractory patients. Such an investigation was started in patient C and the results up till now are summarized in Table IV. Improvement in B_{12}-absorption after oral treatment with one of the preparations would have been reflected in an increased serum B_{12}-level. The first combination tested consisted of the same amount of purified hog intrinsic factor as normally present in 1 U.S.P. unit, together with 5.8 μg of Vit. B_{12}-activity in the form of dried liver. Since this combination had already to be given in the form of 9 tablets per day, owing to the low B_{12}-activity of dried liver, increasing further the amounts of dried liver did not seem desirable.

The serum B_{12}-level after 16 weeks of daily treatment (tablets R.P. 1693) indicated that the absorption of the cobalamins from liver had not improved. The next series of tablets (R.P. 1884) contained only one third of the amount of intrinsic factor concentrate with the same amount of liver, because it seemed possible that the ratio of B_{12} to intrinsic factor in the first oral dose had not been optimal (normally 15 μg cyanocobalamin in 1 U.S.P.-unit). After 9 weeks of treatment no sign of improved absorption was seen here either, and so we changed over to a third type of tablets (R.P. 1891) administered daily. This time the liver was combined with the starting material normally used for the preparation of hog intrinsic factor as used for Bifacton, *viz.* lyophilized hog pyloric mucosa.

After 9 weeks we came to the conclusion that this combination did not improve the resorption either. Although we had planned to continue this investigation by administering dried liver + dried total hog stomach, because this combination would be comparable to the earlier preparations used for the oral treatment of pernicious anaemia patients, the condition of the patient did not allow continuation of the investigation.

The U.E.T. performed at this stage showed that the patient was truly refractory. Apparently the refractoriness of patient C prevented the absorption of the cobalamins of dried liver when the latter was combined with the hog intrinsic factor preparations mentioned above.

KILLANDER[9] has also investigated the effect of oral administration of "cruder" preparations to refractory patients. In one patient a daily oral dose of dried liver containing 16 μg of Vit. B_{12}-activity, together with the equivalent of 1 U.S.P. unit of purified hog intrinsic factor as present in Bifacton, gave no increase in serum B_{12}-level after 3 weeks of treatment, but dried liver + total hog pylorus (15 g/day) gave

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a definite response, resulting in an increase in serum B_{12}-levels of up to more than 100 $\mu\mu$g/ml. Cyanocobalamin + lyophilized total hog pylorus gave no result in his experiments in 3 refractory patients.

BERLIN *et al.*[10] found a response to liver + total stomach in 1 out of 10 cases (1 month treatment). The information at present available does not warrant any definite conclusions, but these first results do not favour the assumption that the use of crude liver or total hog pyloric mucosa improves the resorption of cobalamins, once refractoriness has developed. Of course, it is impossible to draw any conclusion as to the probability whether or not these cruder preparations would give rise to refractoriness, because it is possible that once refractoriness has developed, the patient remains sensitive to hog intrinsic factor (antigenic properties?) or the accompanying substances for a considerable time.

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SUMMARY

In a group of 15 patients with pernicious anaemia, 3 patients were found, who did not react, or no longer reacted to oral therapy with Vit. B_{12} + hog intrinsic factor (refractory pernicious anaemia). This investigation was performed with vitamin B_{12}–^{58}Co, using the urine-excretion test (U.E.T.-SCHILLING) without and with intrinsic factor (I.F.) The U.E.T. without I.F. gave a urine excretion of 6.5–30% ^{58}Co of the oral dose in normal cases; in pernicious anaemia 0–4% was found. Adding intrinsic factor (U.E.T. + I.F.) the urine excretion amounted to 6.2–20%. In the 3 patients with refractory pernicious anaemia no increase of ^{58}Co-excretion in the urine could be demonstrated in the U.E.T. with I.F. Using normal neutralized gastric juice, excretion was increased in one case to 18.8%. The possible influence of the microbial flora on this disturbed resorption was investigated by sterilizing the gastro-intestinal tract (Neomycin) and repeating the U.E.T. with I.F. No improvement could be demonstrated. Variation of the ratio liver B_{12}/intrinsic factor, did not lead to any better result. It is assumed that the hog intrinsic factor concentrates may, in some cases, possess antigenic properties.

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ACTION OF ENZYMES IN PRESENCE OF CERTAIN HORMONES
I. URINE RIBONUCLEASES*

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INTRODUCTION

In an investigation on the specific enzymic activities[1, 2] ribonuclease (RNase) was found to consist of more than one specific enzyme. Ribonuclease was separated into two[3, 4] and resolved[5] into four enzymically specific components.

Being intracellular and secretory enzymes, any ribonuclease excreted in the urine could be a measure of cellular breakdown and indicate the cellular metabolic rate. As to its biological activities, both ribonuclease in the placental extract and crystalline ribonuclease were found to inhibit the growth of transplantable rat carcinoma and spontaneous mice mammary tumors[6].

The menstrual cycle is characterized by alternating periods of rapid cell growth and involution of genital tissues. It affords an excellent opportunity for the study of fundamental chemical and enzymic aspects of cellular metabolism in response to hormonal stimulation occurring normally.

The above observations led us to attempt the characterization of ribonucleases excreted in the urine of normal persons. In this communication we report on specific ribonucleases present in the urine. The effect of magnesium ion and sex hormones on the activities of crystalline ribonuclease is studied. Optimum pH and heat stability, as well as the variation in the amount and in the specific activity of urine ribonucleases during the menstrual cycle are determined.

EXPERIMENTAL

Normal human urine was collected in 24-h samples at various periods of the menstrual cycle. The samples were frozen directly and 25-ml aliquots were lyophilized to dryness and the dried material extracted with 6 ml 0.5 *N* sulfuric acid for 1 h. The supernatant was divided into 3-ml fractions (A and B). Fraction A was adjusted to pH 4.5 and used in the determination of acid RNase[1]. Alkaline RNase activity was obtained from fraction B after adjusting its pH to 7.8. As soon as the urine was collected it was deep-frozen and directly lyophilized. All procedures were carried out under aseptic sterile conditions. We are fully aware of the bacterial flora of the vagina. The dried urine powder was tested for bacterial growth, in no case was growth noted. The urine itself after collection showed a very low bacterial count and in many cases it was negative. Because of the proportionally large volume used, any bacterial ribonuclease could be assumed to be negligible. Factors caused by severe bacterial infection or by abnormal bacterial flora in the genito-urinary track, which might affect

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urinary ribonuclease specificity will be dealt with in a forthcoming research protocol.

Ribonuclease action on yeast ribonucleic acid (RNA)[7, 8] was carried out at pH 4.5 using phosphate buffer (for acid RNase) and at pH 7.8, using phosphate buffer (for alkaline RNase). The enzymic activity was determined by estimating spectrophotometrically[9] the quantity of acid-soluble compounds produced. The action of these enzymes was followed in the presence and absence of magnesium ion ($2 \cdot 10^{-3} M$ Mg).

Chromatograms of the digests of RNA with each of the urine enzymes, acid RNase or alkaline RNase, were developed by the descending technique in two dimensions, using the chromatographic solvents described earlier[10]. The nucleotide areas were located and eluted and the concentrations determined as described in a previous communication[1].

Determination of urinary ribonuclease activity and enzymic specificity

To 1 ml 0.01% solution of the dried urine powder, was added 0.5 ml of 1% ribonucleic acid (RNA) solution and phosphate buffer to a total volume of 2 ml. 0.005 ml chloroform was added for bacteriostatis. After incubation at 37° for 30 min, 0.25-ml aliquots were applied on filter paper for two-dimensional chromatography. To the remaining solution an equal volume of 10% perchloric acid was added. Solutions were refrigerated for 2 h, centrifuged, and the optical density at 260 mμ determined in a model DU Beckman spectrophotometer. Controls consisted of RNA alone exposed to identical conditions.

The chromatograms were developed in two dimensions as described in earlier communications [5–8, 10]. The mononucleotides were located by ultraviolet photography, cut and eluted in 0.01 *N* HCl. The ultraviolet spectrum and the quantitative amount of each of the mononucleotides was determined in a model DU Beckman spectrophotometer.

The above procedure was used to determine the enzymic activity of unheated and heated dried urine powder, in presence and in absence of Mg, at pH 4.5 and 7.8. The same technique was used in obtaining the optimum pH activity of each of the ribonucleases.

RESULTS

In normal human, female urine, ribonuclease was found in free and combined forms. The free enzyme contributed half of the total RNase excreted into the urine. The conjugated RNase was inactive. Treatment of the latter with 0.5 *N* sulfuric acid, resulted in the liberation of the active enzyme (Table I).

TABLE I

URINARY RNase IN 24-h SAMPLES

	RNase activity μg
1. RNase of dried urine	760
2. RNase of dried urine after dialysis	850
3. RNase of dialysed and lyophilized urine	875
4. RNase of step 3. treated with 0.5 *N* H_2SO_4	1650
5. RNase of step 4. fractionated with ammonium sulfate	1520

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The amounts of both free and total RNase gradually increased during the first eleven days of the menstrual cycle. The concentrations in the 24-h urine samples, collected on the 11th day were 760 and 1650 μg of free and total RNase respectively. A gradual decrease occurred after the 11th day until the 17th day, where concentrations dropped to 90 and 785 μg of the free and combined enzymes respectively. A sudden increased value for both enzymes was observed on the 19th day, whereafter the value decreased until the 28th day.

TABLE II

FREE AND TOTAL RNase IN 24-h URINE SAMPLE VARIATIONS DURING THE MENSTRUAL CYCLE

Day of cycle	*Free RNase* μg	*Total RNase* μg	*Estrogen** *I.U.*
1	85	188	100
3	100	225	115
5	109	286	185
7	150	442	215
9	325	942	460
11	760	1650	685
13	522	1375	550
15	125	1100	430
17	90	785	350
19	169	1485	590
21	95	1362	615
23	82	1100	415
25	76	425	275
27	57	315	185
28	53	275	100

* *cf.* ref. [11, 12].

Table II present variations in the amounts of free and total RNases as compared to variations in the amounts of free and total RNases as compared to variations of estrogens in the urine.

On the 27th day, 109 μg free RNase was excreted per l (*i.e.* 109 mμg/ml) of urine at 7.00 a.m. The amount of the enzyme increased gradually to 216 mμg/ml at 12.00, decreased to 69 mμg/ml at 11.00 p.m., then increased to 117 mμg/ml at 7.00 a.m. next morning (Table III).

TABLE III

FREE AND COMBINED RNase PER ml OF URINE VARIATIONS DURING 24 HOURS

Time	*Free RNase* mμg/ml urine	*Total RNase* mμg/ml urine
7.00 a.m.	109	439
9.00 a.m.	127	383
12.00	216	476
11.00 p.m.	69	552
7.00 a.m.	117	470

Urine samples (2 l) were collected separately on the 11th and 21st day of the menstrual cycle (S_{11} and S_{21}). The samples were directly lyophilized to dryness, extracted with 0.5 *N* sulfuric acid and fractionated with ammonium sulfate. The fractions obtained between 0.6 and 0.8 ammonium sulfate saturation were dialyzed

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TABLE IV

OPTIMUM pH ACTIVITY FOR URINE RNases*

pH	*Sample on 11th day*	*Sample on 21st day*	pH	*Sample on 11th day*	*Sample on 21st day*
3.82	0.422	0.410	6.47	0.408	0.465
4.20	0.328	0.422	6.81	0.432	0.510
4.60	0.525	0.375	7.17	0.372	0.552
5.00	0.362	0.375	7.73	0.436	0.470
5.28	0.386	0.458	8.04	0.365	0.502
5.90	0.394	0.465			

* Results are reported in optical density (O.D.) readings, using KUNITZ'[9] spectrophotometric technique for RNase activity.

TABLE V

ACTION OF TEMPERATURE AND MAGNESIUM ON URINE RNases

*Day**	*mμg RNase equivalence at* 0°	60°	80°	100°
S_{11}	195	185	120	110
S_{11} + Mg	173	75	85	70
S_{21}	146	105	75	15
S_{21} + Mg	185	158	115	65

S_{11} and S_{21} are the urine samples collected on the 11th and on the 21st day of the cycle.

for 48 h at 0° and lyophilized to dryness. These two products were used for the characterization of the enzymes present.

The optimum activity of S_{11} was at pH 4.6, while that of S_{21} was at pH 7.17 (Table IV). Sample S_{11} was inhibited by Mg. Heating S_{11} resulted in a progressive inactivation with rise in temperature. Heating at 100° for 15 min produced 43% decrease in activity. Sample S_{21} was heat-labile and only 10% of the original activity was recovered after exposing the enzyme to 100° for 15 min (Table V). This enzyme was activated by Mg.

Fig. 1 present two-dimensional chromatograms of yeast RNA after the action

TABLE VI

SPECIFIC ACTIVITY: ACTION OF URINE RNases ON YEAST RIBONUCLEIC ACID

	Uridylic acid	*Cytidylic acid*	*Guanylic acid*	*Adenylic acid*
	in mmoles per 100 mmoles			
S_{11}	18.8	16.9	1.8	0.0
S_{21}	13.5	12.8	9.5	2.5
S_{11} (60°)	18.7	16.8	1.2	0.0
S_{21} (60°)	13.4	12.7	9.3	0.2
S_{11} (80°)	18.5	16.6	0.2	0.0
S_{21} (80°)	13.3	12.5	3.1	0.0
S_{11} (100°)	18.0	16.4	0.0	0.0
S_{21} (100°)	13.0	12.2	0.5	0.0

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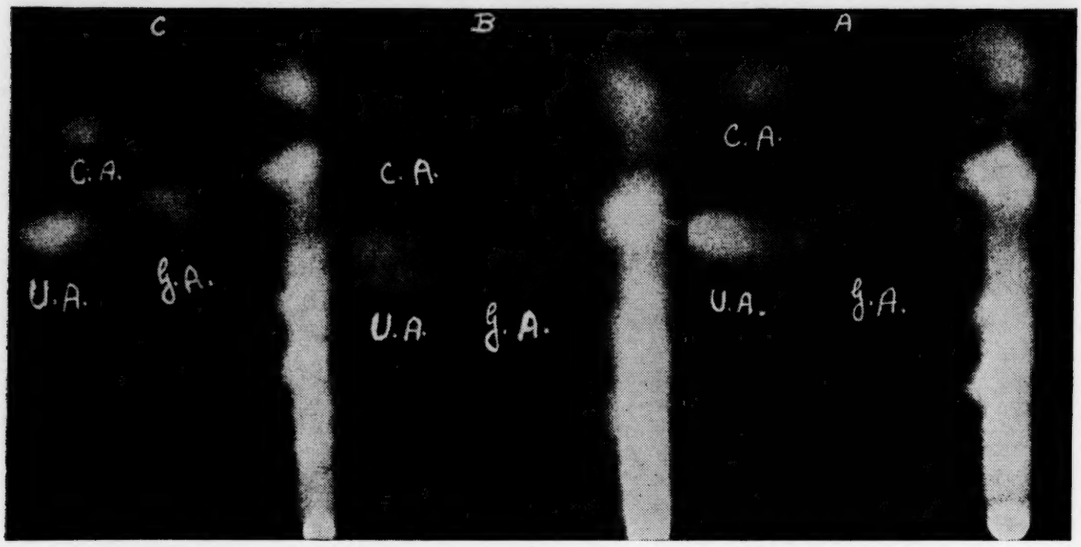

Fig. 1. Two-dimensional chromatograms of yeast ribonucleic acid (RNA) after the addition and action of urine ribonucleases (S_{11} and S_{21}). (A) Yeast RNA after incubation with S_{11} (100°); (B) Yeast RNA after incubation with distilled water (control); (C) Yeast RNA after incubation with S_{21} (100°). U.A., G.A. and C.A. are uridylic, guanylic and cytidylic acid respectively.

of urine ribonucleases (S_{11} and S_{21}). The latter enzyme showed remarkable synthetic activities. Urine ribonucleases samples S_{11} and S_{21} showed different specific activities, when incubated with RNA. The resulting products (pyrimidine and purine nucleotides) are presented in Table VI. After exposure to 100° for 15 min S_{11} liberated 18.0 and 16.4% of uridylic and cytidylic acid respectively. No purine nucleotides were found. Under similar conditions S_{21} liberated 13.0 and 12.2% of uridylic and cytidylic

TABLE VII

CRYSTALLINE RNase ACTIVITY IN THE PRESENCE OF ESTROGENIC HORMONES*

Hormones	*RNase preincubated with*	*RNA preincubated with*
H_2O**	100	100
Diethylstilbesterol	125	44.5
Progesterone	92.8	49.7
Estradiol	94.7	47.7
Estrone	98.0	45.4
Androsterone	91.3	43.8
Testosterone	94.0	50.5

* Results are presented in % of RNase activity in distilled water.
** H_2O: Distilled water was used in the controls to replace the hormone.

acid respectively. Only 0.5% of guanylic acid was detected. Crystalline RNase, when incubated with diethylstylbesterol, was 25% activated. Incubation of crystalline RNase with progresterone, estradiol, estrone, androsterone and testosterone resulted in a slight inhibition of the enzyme.

The above six hormones formed ribonucleates when incubated with RNA. These six different hormone-ribonucleates showed greater resistance to RNase action, and are less soluble than the RNA before treatment (Table VII).

Acid and alkaline RNase activities were found in the sulfuric acid extract of dried urine collected on various days during the menstrual cycle. Aliquots of the sul-

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furic acid extracts were adjusted to pH 5.5 and 7.5. Heating at 60°, 80° and 100° showed that both RNase activities (acid and alkaline) were thermostable. Magnesium ion partially inhibited the unheated acid RNase, but slightly activated the alkaline RNase. Magnesium ion partically inhibited all the heated fractions (60°, 80° and 100°) of both acid and alkaline RNase. Alkaline RNase was thermostable when heated up to 100° for 10 min. Acid RNase heated at 60° for 10 min showed a slight increase in enzymic activity. Heating at 80° for 10 min decreased the enzymic activity, but heating at 100° for 10 min resulted in increased activity. That is, greater enzymic activity was obtained from the fraction heated to 100° then from the unheated fraction (Table VIII).

TABLE VIII

URINE RNase ACTIVITY*

	Alkaline RNase	*Acid RNase*
Urine RNases	0.890	0.818
Urine RNase + Mg	0.900	0.748
Urine RNase (60°)	0.900	0.842
Urine RNase (60°) + Mg	0.860	0.750
Urine RNase (80°)	0.900	0.792
Urine RNase (80°) + Mg	0.885	0.605
Urine RNase (100°)	0.885	0.900
Urine RNase (100°) + Mg	0.805	0.808

* Data are reported in optical density (O.D.) units at 260 mμ, using model Beckman DU spectrophotometer.

The amounts of acid RNase gradually increased from the 1st to the 6th day of the menstrual cycle and gradually decreased between the 19th and 28th day. Alkaline RNase values increased during the first three days, then decreased on the 6th day. A gradual decrease in the alkaline RNase activity was found between the 19th and 27th day, followed by an increase on the 28th day (Table IX).

The effect of magnesium ion on the different enzymic activities found in the dried urine aliquots collected during the menstrual cycle is presented in Table X.

TABLE IX

URINE RNase ACTIVITY DURING THE MENSTRUAL CYCLE*

Day of cycle	*Acid RNase*	*Alkaline RNase*
1	1.180	1.350
3	1.350	1.380
6	1.360	1.230
16	0.600	0.750
19	0.900	0.835
26	0.820	0.820
27	0.755	0.790
28	0.690	0.840

* Data are reported in optical density (O.D.) units at 260 mμ using model Beckman DU spectrophotometer. Optical density of the RNA solution (control) after acid treatment was 0.600 at 260 mμ.

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TABLE X

ACTION OF MAGNESIUM ION ON URINE RNase ACTIVITIES DURING THE MENSTRUAL CYCLE*

Day of cycle	*Acid RNase*	*Alkaline RNase*
1	1.350	1.333
3	1.410	1.310
6	1.350	1.380
16	0.635	0.715
19	0.840	0.808
26	0.860	0.755
27	0.790	0.785
28	0.800	0.850

* Data are reported in optical density (O.D.) units at 260 mμ using model Beckman DU spectrophotometer. Optical density of the RNA solution (control) after acid treatment was 0.600 at of 260 mμ.

DISCUSSION

Ribonucleases are highly specific phosphodiesterases, which hydrolyze ribonucleic acids, certain ribonucleotides[13], synthetic ribonucleotide-P-esters[14], and a natural polymer of ribosephosphoric acid[15]. Depending on the pH for optimum activity, they are divided into acid RNase and alkaline RNase.

In our present studies, we observed the presence of free and combined RNase. The latter was found inactive and treatment with sulfuric acid liberated the active enzyme. The continuous presence of urea in the urine might have effected a separation of active RNase from an inactive conjugated product, presumably by the rupture of certain weak bonds between the enzyme and the attached product.

PIROTTE AND DESREUX[16] have detected an inhibitor for RNase in the supernatant fraction prepared from guinea pig liver. Several other inhibitors for RNase have been described[17–19]. In certain cases, heparin could have been the inhibitory factor[20], while in others the inhibitory effect was attributed to a heparin–lipoprotein complex[21, 22]. The presence of estrogens in the urine and the relationship between free and total ribonucleases with the estrogen excreted during the menstrual cycle (Table II), suggested the possible presence of hormone-conjugated products.

The existence of more than one intracellular RNase in certain tissues was shown by many investigators[23, 24]. They have described one RNase with an optimum pH activity at 5.8 and another at pH 7.8. Our studies indicated the presence of two RNases in the urine of normal persons, one with an optimum pH activity at 4.60 and the other at pH 7.17.

Acid RNase obtained from urine showed an increase, a decrease and a noticeable increase in enzymic activity, when heated at 60°, 80° and 100° respectively (Table VIII). This variation might be due to either the presence of an inhibitor, which is extracted by the sulfuric acid, or to different protein entities[1], which result from modified enzymically active protein molecules[25], produced by the heating effect in the presence of sodium sulfate. The variation was not observed when the salt-free enzymes (S_{11} and S_{21}) were exposed to similar experimental conditions (Table V).

Many factors, such as diet, liquid intake, physical and sexual activities, might influence the quantity and specificity of urine ribonucleases (Table III). Variation in RNA components of the human endometrium[26] were noted as the cycle passes from the proliferative to the late secretory and menstrual phases. TELFER[27] showed a

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prompt and extensive synthesis of uterine RNA by administering estradiol to the adult castrated rat.

Urine RNase activities showed comparable variations as the menstrual cycle passed through its different stages. The RNase activities increased progressively from the end of the menstrual phase, then decreased to reach minimum on the 15th day, increasing again to a maximum and decreasing once more throughout the menstrual phase. The relationship between estrogen excretion and the RNase activities, as well as the effect of estrogens and other sex hormones on RNase enzymic activities are presented in Tables II and VII.

CONCLUSION

The presence of intracellular enzymes in urine of normal persons could be an indication of cellular breakdown. In certain physiological disorders of abnormal cellular metabolism, *i.e.* in malignancy or cancer cell growth, possible variations in amount of the urine ribonucleases are expected. Abnormalities in hormone concentrations as well as disorders in the menstrual cycle could be revealed by urine ribonuclease concentrations. Further studies are in progress.

SUMMARY

Acid and alkaline ribonucleases were found in 0.5 *N* sulfuric acid extracts of dried urine. The effect of magnesium ion and heat on the activities of these urine enzymes was studied. The variation in the amount of the urine ribonucleases during the menstrual cycle was demonstrated. The clinical evaluation of these findings is discussed.

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SERUM DEOXYRIBONUCLEASE I AND II IN PATHOLOGIC CONDITIONS OTHER THAN PANCREAS DISEASES

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An increase of serum deoxyribonuclease I (DNase I with pH optimum 7.2) has been constantly reported in acute pancreatitis, especially in the hemorrhagic form[1]. This increase has been related to the high DNase I content of the pancreas and to its release into the blood stream following necrosis.

Organs other than pancreas have a very low or no detectable DNase I content[2-4]. However, variable amounts of another DNase, with pH optimum 5.6[2, 5]—DNase II—have been found in these tissues.

The two enzymes differ for additional properties other than pH: DNase II does not require Mg^{++} as an activator, and no physiologic inhibitors have been so far detected. Moreover, the products of hydrolysis released from the substrate (mono- and polynucleotides) are different for the two DNases[6-9].

All organs contain DNase II in variable amounts; the highest levels have been found in spleen, liver and kidney. It is also present in pancreatic tissue but it is not excreted with pancreatic juice. This suggests that the two enzymes play a different physiologic role: DNase I is concerned with the digestion; while inhibited in cells, it acts when released as an external secretion. In contrast, DNase II is directly related to the metabolism of deoxyribonucleic acid, and therefore to the mitotic activity of the cells.

Since both enzymes have been found in human serum[10-12] their behavior in different pathological conditions may be of interest. Furthermore, from a general standpoint, this investigation is warranted by the valuable results obtained in the clinical field with the determination of some serum enzymes[13-15].

The present paper reports the results of determinations of serum DNase I and II in normal subjects and in a variety of pathologic conditions, other than pancreatic diseases.

METHODS

DNase activity may be expressed in different ways according to the method of determination. By quantitating the amount of the pentose released from substrate during hydrolysis, enzymic activity may be expressed as μg of deoxyribose.

To determine DNase I activity we used the following procedure[10]: Three 10-ml centrifuge tubes are labelled A, B and C; 1 ml of the serum to be tested is pipetted into A and C; 1 ml of veronal buffer (pH 7.4) is added to each tube, and subsequently, 0.5 ml of DNA (Schwarz, Mount Vernon, N.Y.) solution (0.6% in 0.05 *M* $MgSO_4$) only to tubes A and B. To tube C, 0.5 ml of twice-distilled water is added. The tubes are incubated in a water bath at 37°; after 4 h 1 ml of serum is added to tube B and the reaction is immediately stopped in all tubes with 0.8 ml of trichloroacetic

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acid (TCA) 3.6 *M*. After shaking and centrifuging, 0.5 ml of the clear supernatant fluid is withdrawn; deoxyribose is assayed according to the classic DISCHE method[16].

For the assay of DNase II acetic buffer (pH 5.6) is used instead of veronal, and the DNA is dissolved in twice-distilled water only.

The details of the procedure are reported in Table I:

TABLE I

	DNase I			*DNase II*		
	A	*B*	*C*	*A*	*B*	*C*
Serum (ml)	1	–	1	1	–	1
Buffer pH 7.4 (ml)	1	1	1	–	–	–
Buffer pH 5.6 (ml)	–	–	–	1	1	1
DNA (ml)	0.5	0.5	–	0.5	0.5	–
H_2O (ml)	–	–	0.5	–	–	0.5
put in a water bath at 37° for 4 h						
Serum (ml)	–	1	–	–	1	–
TCA (ml)	0.8	0.8	0.8	0.8	0.8	0.8
shake, centrifuge and add to 0.5 ml of clear supernatant fluid						
Dische (ml)	1	1	1	1	1	1
put in a boiling water bath for 10 min and then add 1.5 ml of sulfuric acid 1.5% in acetic acid.						

On a spectrophotometer (at 625 mμ wavelength) A and B are examined with C as blank and the extinction values are recorded. The number of μg of deoxyribose released by the enzymic reaction is obtained from the difference between A and B; this correction is necessary in view of the fact that commercial preparations of deoxyribonucleic acid contain variable amounts of depolymerized products.

TABLE II

SERUM DNase I ACTIVITY

Subjects	*No.*	*Mean*	*Range*	*Deoxyribose (μg)*													
				0–10		*10–20*		*20–30*		*30–40*		*40–50*		*50–60*		*60–70*		*>70*	
				n°	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%
Normal controls	21	20.8	0–59.4	6	29	9	43	1	5	2	10	1	5	2	10	–	–	–	–
Malignant disease	46	7.6	0–59.4	36	78	6	13	1	2	–	–	1	2	2	4	–	–	–	–
Liver disease	37	19.3	0–99.0	18	49	8	22	4	11	1	3	2	5	–	–	1	3	3	8
Misc. pathologic conditions	13	8.0	0–36.3	9	69	2	15	–	–	2	15	–	–	–	–	–	–	–	–

TABLE III

SERUM DNase II ACTIVITY

Subjects	*No.*	*Mean*	*Range*	*Deoxyribose (μg)*													
				0–10		*10–20*		*20–30*		*30–40*		*40–50*		*50–60*		*60–70*		*>70*	
				n°	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%	*n°*	%
Normal controls	20	18.2	0–39.6	8	40	8	40	1	5	3	15	–	–	–	–	–	–	–	–
Malignant disease	46	9.3	0–67.2	29	63	12	26	4	9	–	–	–	–	–	–	1	2	–	–
Liver disease	37	12.9	0–125.4	23	62	10	27	1	3	1	3	–	–	–	–	–	–	2	5
Misc. pathologic conditions	13	4.0	0–23.2	11	85	1	8	1	8	–	–	–	–	–	–	–	–	–	–

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The calibration chart may be obtained by using pure deoxyribose. Enzymic activity is expressed as μg of deoxyribose released from 1 ml of serum. By using progressive dilution of pure DNase I (Mann) we found that 100 μg of deoxyribose are released by a solution containing 2.5 μg of DNase/ml.

DNase I and II have been measured in the sera of 117 subjects; 21 were normal controls, students and physicians, 46 were patients with malignant proliferative disease (27 untreated carcinomas, 8 carcinomas after XR treatment, 11 leukemias and Hodgkin's disease), 37 were patients with liver disease (12 cirrhosis, 19 obstructive jaundice and 6 viral hepatitis); 13 additional patients had various diseases (gastric ulcer, congestive heart failure, myocardial infarction).

RESULTS

The results are reported in Tables II and III and in Figs. 1 and 2. The following conclusions can be drawn:

1) With simultaneous determinations no correlation can be found between DNase I and DNase II activity, in either normal or pathological conditions.

2) There is a wide range of values in all groups.

3) In a large number of patients with malignant diseases low values both of DNase I and DNase II have been observed. Whereas only 28.6% (DNase I) and 40% (DNase II) of normal controls show values between 0 and 10 μg, up to 78.2% (DNase I) and 63% (DNase II) of the cases with cancer are found in this low range of activity. As to the DNase I the difference has been proved to be statistically significant (χ^2 = 15.387; P 0.001). No definite conclusions can be drawn in regard to DNase II, the observed χ^2 value having a P between 0.10 and 0.05.

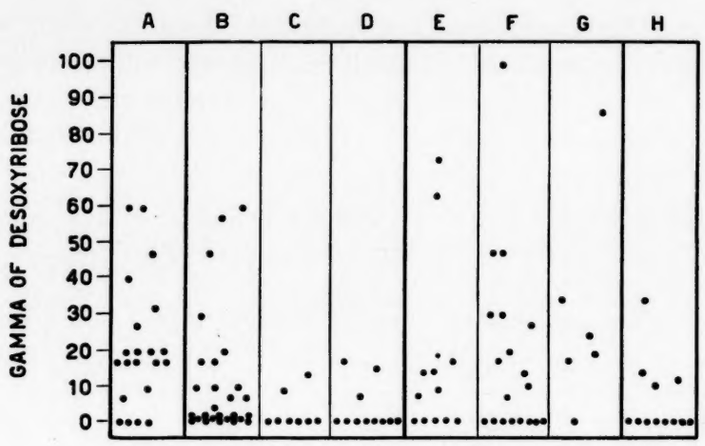

Fig. 1. DNase I activity (A = normal controls; B = carcinomas; C = carcinomas after XR treatment; D = leukemias and Hodgkin's diseases; E = cirrhosis; F = obstructive jaundice; G = hepatitis; H = various pathologic conditions).

4) Only occasionally very high values have been found (above the normal limits of 60 μg of deoxyribose for DNase I and 40 μg for DNase II). These were consistently in patients with a very severe degree of hepatic damage.

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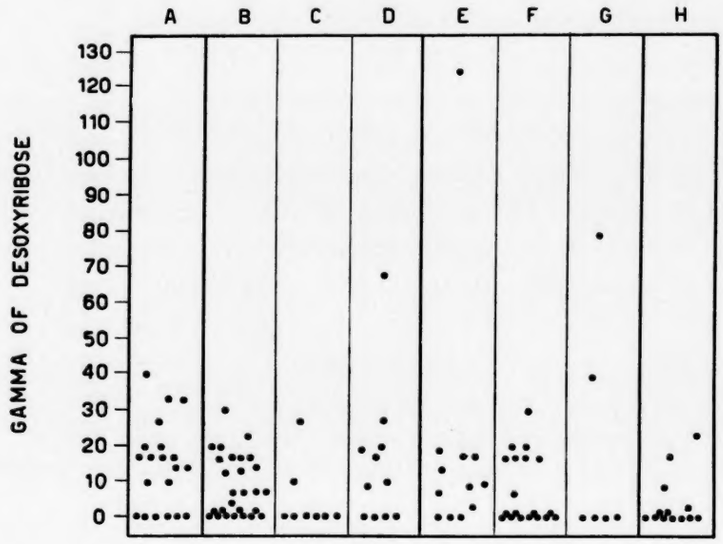

Fig. 2. DNase II activity (A = normal controls; B = carcinomas; C = carcinomas after XR treatment; D = leukemias and Hodgkin's diseases; E = cirrhosis; F = obstructive jaundice; G = hepatitis; H = various pathologic conditions).

COMMENT

A significative increase of pancreatic DNase activity has recently been shown in acute pancreant as well as in pancreatic necrosis experimentally induced with ethionine[1] or COXSACKIE virus[17]. The present data show that increased DNase I and DNase II activities cannot be considered characteristic of any other disease. Occasional high values of both enzymes have been observed in a few cases of advanced liver insufficiency with severe jaundice.

According to KURNICK AND CARRERA[18] the liver might withdraw the enzyme from the blood stream and thus regulate its plasma level: a decreased clearing function would thus follow liver impairment. They observed also that serum DNase (DNase I) is considerably elevated in parenchymatous liver disease but not in obstructive icterus in man. The present data do not substantiate these results. Therefore the determination of serum levels of these enzymes would not seem to be as reliable a guide for the evaluation of liver damage as the use of other enzymes (phosphatases, transaminase, aldolase, phosphoisomerase, phosphoglyceromutase).

Significantly low values of DNase I have been observed by WROBLESKI AND BODANSKY[19] in 50 patients with malignant diseases as compared with normal individuals of the same age; no significant changes have been observed by KURNICK[11]. The present results show a statistically significant decrease of DNase I. Since many of these patients have very low values, further investigation is needed to ascertain the possible relationship of level with size and type of tumor.

SCHREIDER *et al.*[10] have reported increased values of both enzymes in children irradiated for extensive tumors. Our limited data on irradiated adult subjects seem to conflict with the previous report but need further study.

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SUMMARY

Serum deoxyribonuclease I and II have been studied in a group of patients with liver disease, carcinomas and miscellaneous conditions. Since both enzymes have been found in human sera, their behavior in pathological conditions may be of interest and eventually of diagnostic value. A wide range of values has been found in all groups with no correlation between DNase I and DNase II. Significantly low values have been found in patients with malignant diseases whereas high values have been occasionally observed in cases with advanced liver insufficiency.

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DETERMINATION OF GLYCERIDES IN BLOOD SERUM

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There exists as yet no specific chemical method for the determination of glycerides in blood serum. The analytical procedure must be directed either towards the molecule as a whole, or towards some part of it: the fatty acids, the ester bond, or the glycerol. With all these analytical procedures for the estimation of glycerides one or several of the other lipid compounds present in an extract of the serum lipids will also be determined. Consequently, the determination of the serum glycerides will require either an indirect type of estimation or isolation of the glycerides from similarly reacting lipids before the estimation.

Indirect analysis requiring the estimation of several lipid compounds for the determination of neutral fat has been carried out in many different ways, owing to the gradual development in lipid chemistry of different analytical methods. The nephelometric technique, involving measurement of the turbidity of aqueous suspensions of the lipids was introduced by BLOOR[1, 2]. BANG[3] proposed his oxidimetric method in 1918. In this method the lipids were oxidized with a known amount of bichromate–sulfuric acid mixture and the bichromate not consumed was titrated. In 1951 BRAGDON[4] converted this method to a colorimetric analysis. Acidimetric titration of the fatty acids after saponification was introduced by STEWART AND WHITE[5] and by STODDARD AND DURY[6]. In 1933 MAN AND GILDEA[7] critically revised and modified the technique for titration of the fatty acids. A gasometric micromethod for the analysis of lipids was proposed by KIRK, PAGE AND VAN SLYKE[8] (1934). The glycerol part of the molecule was estimated by BLIX[9, 10] (1937, 1940) and by VORIS *et al.*[11] (1940). The amount of total esterified fatty acids has also been determined by analysis of the ester bond. BAUER AND HIRSCH[12] (1949) converted the fatty acid esters into the red-coloured hydroxamic acids. A more exclusive approach was suggested by FREEMAN *et al.*[13] (1953). These authors measured the absorbancy of the ester bond at 5.8 μ by infrared spectrometry.

The development of chromatographic methods for the separation of the various lipid compounds by the extensive work of TRAPPE[14, 15] (1940) and later of BORGSTRÖM[16] (1952), has now made it possible to analyse neutral fat in a more exact manner, avoiding the errors of the earlier methods. The chromatographic technique of BORGSTRÖM has been applied for the determination of glycerides by MEAD AND FILLERUP[17] (1954) and by LINDGREN *et al.*[18] (1955).

The aim of the present study has been to develop as specific a method as possible for the estimation of the glycerides in serum. Furthermore, the method should be suitable for the routine analysis of many samples. The method has in its present form been in use for more than two years. The principal features and some preliminary results have been described earlier[19].

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Principally the method is based upon the determination of glyceride glycerol. The glycerides are first separated from the phospholipids by chromatography on silicic acid. After saponification the glycerol is determined by estimation of the formaldehyde formed by periodic acid oxidation.

EXPERIMENTAL

Reagents

Organic solvents. Chloroform, methanol and light petroleum ether of reagent grade were all distilled in all-glass apparatus before use. Ethanol (95%, A. B. Svenska Vin- och Spritcentralen) was used without further purification (about 1 bottle in 10 to 20 gave too high blank values).

Silicic acid. For chromatographic purposes Baker's "Silicic acid, Powder" was used. Before use it was activated for about 12 hours at 120°.

0.02 M sodium periodate. 1.15 g of periodic acid dissolved in 225 ml of distilled water and neutralized with 0.2 *N* sodium hydroxide using methyl red as indicator.

0.2 M sodium arsenite. 1.8 g of sodium hydroxide and 4 g of arsenious acid dissolved in 200 ml of distilled water.

Chromotropic acid reagent. 300 ml of concentrated sulfuric acid are mixed with 150 ml of distilled water and cooled. 1 g of chromotropic acid (4,5-dihydroxynaphthalene-2,7-disulphonic acid disodium salt) Eastman-Kodak or B.D.H., is dissolved in 100 ml of distilled water, filtered and added to the diluted acid. The reagent has to be stored in a dark bottle.

Formaldehyde. Hexamethylenetetraamine was purified by sublimation under reduced pressure. Formaldehyde was then evolved by acid hydrolysis according to the directions of McFADYEN[20].

Glycerol. The concentration of glycerol in reagent grade samples was calculated from the gravity.

Glycerides. Triolein (Merck) and tripalmitin (B.D.H.) were purified by recrystallization and or chromatography on silicic acid. Pure samples of 1,3-dipalmitin and monoolein were kindly supplied by DR. B. BORGSTRÖM, Lund.

Procedure

Extraction. 8 ml of methanol are placed in a 25-ml volumetric flask and 1 ml of serum is pipetted into the methanol. 8–10 ml of chloroform are added, the content mixed and brought just to the boil on the steam bath with agitation to prevent bumping. After cooling the flask is made up to volume, the suspension mixed, and rapidly filtered. 20 ml of the filtrate are transferred to a separatory funnel and shaken for 30 sec with 8 ml of saline. The phases are then allowed to separate for some hours, preferably overnight. The chloroform phase is drawn off and the funnel rinsed with about 1 ml of chloroform. The chloroform extract is evaporated to a volume of about 2 ml and subjected to chromatography on silicic acid.

Separation of phospholipids from glycerides by chromatography. 400 mg of activated silicic acid mixed with 200 mg of Hyflo Supercel are packed on columns with an internal diameter of 7 mm. When many columns are to be prepared silicic acid and Hyflo are slurried in light petroleum ether. 4 ml of the slurry, containing 400 mg of silicic acid and 200 mg of Hyflo, are pipetted into each column. When the adsorbent has

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settled it is washed with chloroform. The sample is than applied, and the glycerides are eluted directly into a 25-ml volumetric flask with chloroform until the flask is filled up to the mark.

Saponification. Of the chloroform eluate, containing the glycerides, aliquots containing 5–20 μg of glyceride glycerol (usually 3 ml) are pipetted into 25-ml flasks for the glycerol determination. The determination is carried out in triplicate or duplicate. The chloroform is evaporated on the steam bath, 1 ml of ethanol and 1 drop of 2.5% potassium hydroxide are added and the flasks shaken. The sample is saponified at 60° for 30 min, acidified with 2 drops of 6% acetic acid and taken just to dryness on a steam bath.

Extraction of the fatty acids. About 10 ml of light petroleum ether are added to each flask and brought to the boil on the steam bath.

Extraction of the glycerol. 1 ml of 0.67 *M* sulfuric acid is pipetted into each flask. The flasks are carefully shaken, and the petroleum ether is decanted off.

Periodic acid oxidation. 0.3 ml of the sulfuric acid solution is pipetted off in duplicate into test tubes 10 × 100 mm. 0.1 ml of sodium periodate is added, the tubes are shaken and allowed to stand for 10 min. After the addition of 0.1 ml of sodium arsenite the tubes are shaken again and allowed to stand for about 5 min.

Chromotropic acid reaction. 2.5 ml of the chromotropic acid reagent are added to each tube, preferably from an automatic pipette. This ought to be done in a room without direct daylight, as the reagent is rather sensitive to light. Accordingly, the racks used are especially constructed to shield the tubes from direct light. After the addition of the reagent the tubes are carefully shaken, and placed in a boiling water bath for 30 min. After cooling to room temperature, the optical density at 570 mμ is read in a Beckman model B spectrophotometer in 1-cm cuvettes. Standard and blank samples are run simultaneously through the whole procedure. Tripalmitin is used as standard.

RESULTS AND DISCUSSION

The present method is, in its final steps, in essence a modification of the method for glycerol determination elaborated by LAMBERT AND NEISH[21] applied to glyceride glycerol. These authors introduced a reduction of the excess of periodate and iodate to iodide by addition of arsenite. In this way the destillation of the formed formaldehyde was eliminated, as iodide does not interfere with the subsequent chromotropic acid reaction.

The colorimetric determination of formaldehyde was performed according to the directions of MCFADYEN[20]. To keep the blank values low, we found it necessary to have as low a salt concentration as possible. Working with a periodate concentration of 0.02 *M* and an arsenite concentration of 0.2 *M* blank values around 0.070 were obtained in 1-cm cuvettes.

The completeness of the periodate oxidation was checked by studying the amount of formaldehyde liberated after different time intervals. As shown in Fig. 1, no formaldehyde was liberated after 7–8 minutes. That the oxidation was not only optimal but also almost complete has been ascertained by comparing the data obtained from the oxidation of glycerol with those obtained from the chromotropic acid reaction with pure formaldehyde. These experiments were performed with ten samples each of

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glycerol and formaldehyde. The results given in Table I show that the glycerol had been oxidized to 97.5% of the theoretical value.

During the work with pure glycerides in the earlier phase of this investigation we found that now and then the values obtained were too high when triolein was used as the test substance. In series of determinations the values were sometimes too high in comparison with those obtained for glycerol only. Experiments with oleic acid added to glycerol showed the same tendency. It was thus possible that the high values might originate from the oleic acid. In order to test this assumption further, spectral

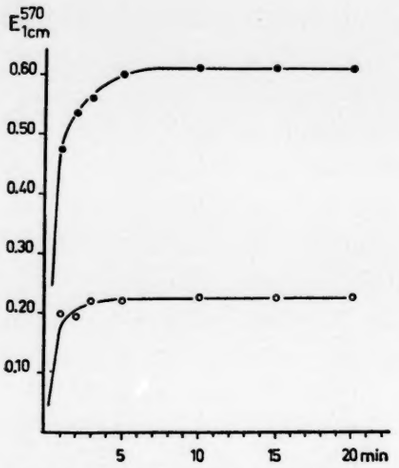

Fig. 1. Periodic acid oxidation of glycerol of different concentrations according to the technique described.

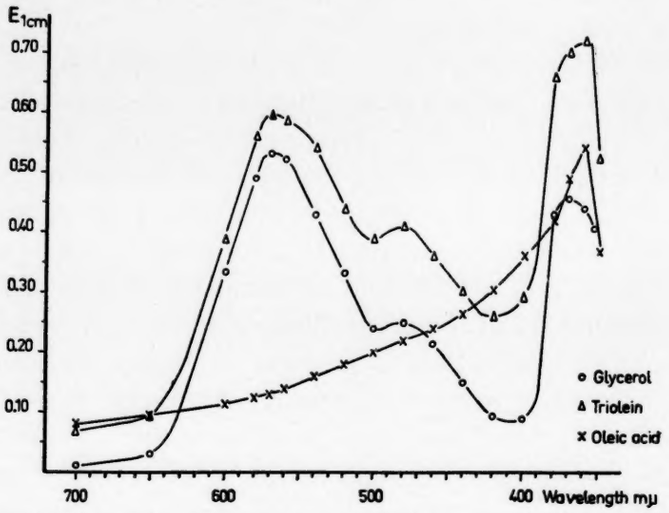

Fig. 2. Spectral curves for equivalent amounts of glycerol, triolein and oleic acid after saponification, periodic acid oxidation and reaction with chromotropic acid.

TABLE I

ESTIMATION OF THE COMPLETENESS OF THE PERIODIC ACID OXIDATION OF GLYCEROL TO FORMALDEHYDE

	Formaldehyde 2.73 μg	*Glycerol* 4.95 μg		*Recovery of formaldehyde*
		Observed	*Calculated from the data of formaldehyde*	
Extinction at 570 mμ	0.495	0.571	0.586	97.5%

curves were registered of the products obtained after the chromotropic acid reaction. Fig. 2 gives the curves for equivalent amounts of glycerol and triolein analysed according to the above procedure omitting the extraction with light petroleum ether. If the spectral curve for triolein is compared to the one for glycerol, it is found that the maxima both at 570 and at 480 mμ are higher and, furthermore, that the peak at 370 mμ has shifted to 360 mμ. These findings can be explained by the spectral curve obtained for oleic acid analysed in the same way. This curve is also given in the figure. Experiments with palmitic and stearic acid did not show this interference. Since the additional step with petroleum ether extraction of the acids after the saponification was introduced, none of the values obtained have been too high.

The close agreement between the values obtained for different glycerides is appa-

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rent in Table II. Here, four glycerides were analysed according to the described procedure including extraction with light petroleum. The figures represent mean values from 8 separate determinations of each compound and have been compared with the value obtained by direct periodic acid oxidation of glycerol.

TABLE II

COMPARISON OF THE EXTINCTION VALUES AT 570 mμ FOR GLYCEROL WITH THOSE OBTAINED FOR DIFFERENT GLYCERIDES WITH THE DESCRIBED PROCEDURE

	Glycerol	*Monoolein*	*Dipalmitin*	*Tripalmitin*	*Triolein*
Amount in μg	3.95	18.6	26.6	44.2	50.9
Extinction	0.469	0.565	0.516	0.590	0.631
Extinction per μmole	10.9	10.8	11.0	10.8	11.0

The linearity of the reaction was studied with varying amounts of different glycerides. The extinction curve was found to be linear for extinction values up to 1.0.

The partition of tri-, di- and monoglycerides between the chloroform phase and the aqueous methanol phase in the washing procedure as well as the recovery of these glycerides after silicic acid chromatography was controlled in two ways. First the partion of pure glycerides was studied and secondly different glycerides were added to primary extracts of serum lipids and the distribution estimated by determination of the amount of glycerides in the chloroform phase after passage through silicic acid. In all cases tri-, di- and monoglycerides were recovered in amounts of more than 95%.

In order to ascertain whether the extraction yield could be increased by refluxing the serum proteins with chloroform–methanol, the following experiments were performed. Three different sera were selected: one normal, one from a case of essential hyperlipaemia, and one from a case of diabetes with chronic pancreatitis. The sera were extracted on the one hand as described above, and on the other by boiling under reflux with chloroform–methanol (1:1) for 2 hours. Four samples were taken from each extract for the determination of glycerides, cholesterol and phospholipids. The results are presented in Table III. The agreement between the two extraction procedures shows clearly that the yield could not be increased by boiling under reflux.

TABLE III

THE EFFECT OF PROLONGED BOILING ON EXTRACTION EFFICIENCY

	Glycerides mmoles/l			*Cholesterol mg/100 ml*			*Phospholipids* mg/100 ml*	
	a	*b*	*c*	*a*	*b*	*c*	*b*	*c*
Extraction with chloroform–methanol as described under procedure	0.92	12.8	6.4	245	905	390	665	400
Extraction with chloroform–methanol by boiling with reflux for 2 hours	0.92	12.8	6.5	235	930	375	660	395

a = normal serum
b = serum from a case of essential hyperlipaemia.
c = serum from a case of diabetes mellitus.
* The sample from the normal case was lost.

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The sera from 29 apparently healthy individuals of both sexes between 20 and 35 years old were analysed. A mean value of 0.87±0.33 mmoles per litre (range 0.34–1.53) was found. The methodical error of a single determination of the serum glycerides was calculated from 121 duplicate determinations ($\sqrt{\Sigma d^2/2n}$) and was found to be ± 0.02 mmoles per litre. The mean value for this series was 1.03 mmoles per litre (range 0.4–2.7). The sensitivity of the technique allows estimation of glycerides in 0.1 ml of serum.

GENERAL DISCUSSION

The method described has certain advantages over earlier conventional methods with regard to specificity. Already in the extraction procedure the water-soluble substances are partitioned into the upper methanol–water phase and removed from the lipids. This permits more careful handling than evaporation and drying of a preliminary lipid extract and then reextraction of lipids with, for instance, light petroleum ether. Furthermore, in the latter procedure non-lipid substances are known to be extracted[22, 23]. The extraction of blood lipids with chloroform–methanol according to FOLCH *et al.*, as well as the washing procedure, has been studied in detail earlier [24–27]. That the extraction is complete or at least that the yield cannot be increased by prolonged boiling under reflux has been shown above. FOLCH *et al.*[27] suggested that the chloroform–methanol extract has to be washed with exactly 0.2 its volume of water. This proportion was said to be critical to avoid emulsions. We have constantly used double the amount of water, *i.e.* 0.4 of the volume of the extract. In this way the chloroform phase was freed from methanol sufficiently to permit chromatography on silicic acid directly without taking the extract to dryness. We found this technique excellent and have never observed any tendency to emulsification. From the experiments with pure glycerides and from the recovery experiments it is also clear that the glycerides are quantitatively recovered in the chloroform phase.

The next step, chromatography on silicic acid, separates the glycerides from the other glycerol-containing lipids, *i.e.* the phospholipids. In this way the error introduced by calculation of phospholipid glycerol from phospholipid phosphorus is avoided. The completeness of the separation under our conditions has been checked by phosphorus analysis of the chloroform eluates. In no case were detectable amounts of phosphorus found after elution of the glycerides from the silicic acid. In addition, according to the figures given by BORGSTRÖM[16], our columns are sufficiently large for phospholipid concentrations of about 1500 mg per 100 ml of serum.

The extraction, after hydrolysis, with light petroleum ether in an acid medium removes lipid compounds of which some might interfere with the subsequent colorimetric analysis. The possible interference of oleic acid has already been discussed above.

The final step is the estimation of the glycerol. This is done by periodic acid oxidation of glycerol to two moles of formaldehyde per mole of glycerol. The formaldehyde is estimated by the chromotropic acid reaction. The behaviour and specificity of this reaction has been studied in detail by MCFADYEN[20] and his directions were followed. Under our conditions the oxidation is optimal and almost complete after 10 minutes. That there are no losses of glycerol or formaldehyde during the procedure is evident from the experiments reported in Tables I and II. The absence after saponification of formaldehyde or other substances which might interfere with the results

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TABLE IV

Author	*Source*	*Glyceride concentration*	*No.*	*Sex*	*Analytical method*	*Glyceride estimation*	*Remarks*
BLOOR[2], 1916	Plasma	110 mg/100 ml 160 mg/100 ml	14 9	♂ ♀	Nephelometric	Total lipids — (0.7 × phospholipids + 0.4 × cholesterol)	
BOYD[28], 1933	Plasma	154 mg/100 ml	8	♀	Bichromate oxidation	Total lipid C — (free cholesterol C + cholesterolester C + phospholipid C)	Requires a constant composition of the cholesterol derivatives and of the phospholipids. Unidentified compounds extracted with petroleum ether and free fatty acids will probably be included
PAGE *et al.*[29], 1935	Plasma	225 mg/100 ml	66	♂	Gasometric for total lipids and cholesterol. Phospholipids from lipid phosphorus	Total lipid C — (free cholesterol C + cholesterol ester C + phospholipid C)	"...the material... may contain besides simple triglycerides unidentified petroleum ether-soluble material of other nature."
BLIX[10], 1940	Plasma	49 mg/100 ml		Both	Glycerol determination	Total lipid glycerol—phospholipid glycerol calculated from lipid phosphorus	Requires a constant proportion of sphingomyelin
PETERS AND MAN[30], 1943	Serum	3.12 mequiv./l	99	Both	Titration of fatty acids	Total fatty acids — (cholesterol ester fatty acids + phospholipid fatty acid)	Requires a constant proportion of sphingomyelin and cholesterol esters. One negative value was obtained.
BRAGDON[4], 1951	Plasma	128 mg/100 ml	12	Both	Bichromate oxidation	Total lipids — (cholesterol esters + cholesterol + phospholipids)	*Cf.* BOYD[28] *
STEWART[31], 1954	Whole blood	87 mg/100 ml	11	Both	Glycerol determination	Correction for phospholipid glycerol from standard curves	

* By refining the extraction procedure using chloroform–methanol and washing this extract BRAGDON obtained lower values than with the above procedure with a mean value of about 100 mg/100 ml (BRAGDON, personal communication, 1958).

was checked by performing the chromotropic acid reaction without periodic acid oxidation. Blank controls performed in this way did not show the presence of interfering or colour-yielding substances.

When using this method the content of glycerides in serum is most conveniently expressed in molar concentration. It has to be kept in mind that the glyceride glycerol might to some extent originate from di- and monoglycerides as well as from triglycerides. In a study of the glyceride composition of human serum it was found that di- and mono-glycerides might contribute up to about 5–10% of the glyceride glycerol[19]. Furthermore, the error introduced by the use of a standard mean molecular weight for the fatty acids is thus avoided.

Some of the more usual and important earlier methods have been collected in Table IV. The results as well as the main technique are included in the table with some comments. All these values were based on a more or less extensive calculation of the glyceride concentration. This must of necessity decrease the accuracy of the determination. Furthermore, it offers no certainty that the calculated value represents the glyceride fraction alone, at least when more unspecific analytical methods are used, as for instance carbon estimation or bichromate oxidation. The titration of fatty acids, on the other hand, might include the free fatty acids in the glyceride value.

The values best corresponding to ours are those obtained by BLIX[10], PETERS AND MAN[30] and BRAGDON[4]. The other values are apparently higher. This might at least partly be ascribed to the lack of accuracy and specificity of the earlier methods, as discussed above. However, no direct comparisons are possible since variables such as age, sex and dietary habits might well influence the level of the serum glycerides.

SUMMARY

A method has been presented for the estimation of the serum glycerides. The basic principle is the determination of the glycerol part of the glycerides after separation of the phospholipids on silicic acid. The method is discussed and compared with earlier methods for the estimation of the glycerides in blood serum.

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ADDENDUM

Since this paper was prepared our attention has been directed to an article by VAN HANDEL AND ZILVERSMIT (*J. Lab. Clin. Med.*, 50 (1957) 152) on the same subject. These authors utilized the principles as described above and earlier[19] by us. However, there are some minor differences which need brief comment. Certainly their extraction and purification procedure using Doucil–chloroform is simpler than our technique and apparently gives a good yield as compared to chloroform–methanol extraction with separation on silicic acid (95, 95 and 98% as judged from three reported experiments on dog plasma). However, our technique was developed for purposes including recovery of all the lipid constituents of a plasma sample (the unesterified fatty acids are quantitatively recovered in the chloroform phase if the washing is done with acid phosphate instead of saline). After saponification no extraction of the fatty acids was found necessary, which conflicts with our experience. This point cannot be evaluated at present. It has been shown that with the technique we used there is no danger of losing glycerol when the ethanol is evaporated after saponification, and an unsaponified blank from each sample has not been necessary, a fact which might be explained by our extraction with petroleum ether after saponification. However, the magnitude of this presaponification blank was not reported. It is of interest that their normal values for 14 young men were very close to the values reported here by us.

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CONTRIBUTION AU DOSAGE DE LA CHOLINE DANS LE SÉRUM SANGUIN

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Le dosage de la choline a fait l'objet de nombreux mémoires. La précipitation préalable de la choline, étant donné l'absence de réaction colorées spécifiques, présente l'avantage de séparer le produit à doser et d'éliminer en majeure partie les substances gênantes. Pour la précipitation on a fait naturellement appel aux agents de précipitation des bases azotées: le chloroaurate, le chloroplatinate, l'iode ioduré, le sel de Reinecke, les acides phospho- et silicotungstiques. Cependant la précipitation de la choline n'est pas toujours quantitative. D'une part le coefficient de solubilité et d'autre part le manque de sensibilité des réactions ou déterminations effectuées sur les précipités mêmes rendent le dosage difficile, voire impossible, lorsqu'il s'agit de doser des quantités particulièrement faibles.

Nous nous sommes proposé de trouver d'une part un réactif de précipitation adéquat et d'autre part de sensibiliser la réaction colorimétrique effectuée à l'aide du sel de Reinecke.

I. LA PRÉCIPITATION DE LA CHOLINE PAR UN POLYHÉTÉROACIDE

Les procédés de dosage à l'aide de ces acides sont peu nombreux[1, 2]. Les précipités obtenus étant relativement solubles, ces méthodes nécessitent des prises d'essai tellement importantes qu'elles ne sont pas applicables en biologie.

Cependant, l'un de nous a signalé antérieurement qu'en solution éthérée, de faibles quantités de bases azotées précipitent par certains acides ou sels inorganiques, alors qu'en milieu aqueux ces derniers sont dépourvus de toute réactivité, – le choix du solvant étant déterminant et la nature de l'agent précipitant d'importance secondaire. L'acide silicotungstique se trouve dans ce cas. Nous avons déterminé d'abord la sensibilité de l'acide silicotungstique vis-a-vis de la choline et constaté qu'en solution aqueuse cette sensibilité atteint à peine 1/3200 et est encore plus faible en milieu acide (acide sulfurique *N*). En milieu alcoolique, la sensibilité atteint 1/8000. Par contre, en milieu éthéré, on obtient encore des solutions opalescentes à la concentration de 1/5,000,000.

Rappelons que pour les A^+, la base ou le sel est dissous dans l'alcool et précipité par une solution éthérée d'acide silicotungstique, la concentration alcoolique finale de la solution éthérée étant maintenue à environ 15%. En solution aqueuse (dans les cas favorables) l'acide silicotungstique fournit avec les A^+ un précipité dont la formule de constitution est habituellement représentée de façon schématique par $SiO_2 \cdot 12WO_3 \cdot 4A^+$*.

* Rappelons qu'il serait préférable de représenter le formule de constitution par $12WO_3 \cdot SiO_4H_4 \cdot 4A^+ \cdot 2H_2O$ ou mieux encore, par $H_8Si(W_2O_7)_6 \cdot 4A^+$ correspondant à un sel $K_8Si(W_2O_7)_6$ qu'il est possible de transformer en sel tétrapotassique: $SiW_{12}O_{42}K_4H_4$. Si l'on concentre une solution de sel neutre additionnée d'un excès d'acide sulfurique, il se forme entre autres un sel tétrapotassique qui donne naissance à un sel tripotassique $SiW_{12}O_{42}K_3H_5$.

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En solution éthérée on obtient un précipité de composition: 1 acide silicotungstique–3 alcaloïdes.

Nous avons cependant constaté que pour de faibles quantités de choline à doser il faut un excès de réactif qui comporte au moins 50 × la quantité théorique si l'on désire obtenir des précipités de composition constante (1/3). Nous avons adopté la technique suivante. La solution alcoolique de choline est évaporée en tube à centrifuger jusqu'à environ 0.2 ml. On ajoute lentement 10 ml de solution éthérée d'acide silicotungstique (5 mg/ml)*. On centrifuge; on décante le liquide surnageant, laisse égoutter et lave 2 × avec 10 ml d'éther contenant 15% d'alcool. On dissout le précipité dans 4 ml d'eau (chauffer légèrement), refroidit la solution et ajoute 2 ml d'une solution d'hydrosulfite de sodium (1 g dans 15 ml d'eau). La coloration se maintient au moins pendant 20 minutes**.

Quantité de choline précipitée mg	*Quantité de choline trouvée par voie colorimétrique mg*
0.20	0.21
0.20	0.19
0.41	0.40
0.61	0.62
0.81	0.80
1.02	1.02
1.22	1.24

Nous avons contrôlé la composition de nos précipités en effectuant la précipitation de 2.9 mg, 2.7 mg et 1.45 mg de chlorure de choline, en recueillant les précipités et en confrontant les résultats obtenus par pesée avec ceux fournis par les mesures colorimétriques effectuées sur ces précipités. Dosages gravimétrique et colorimétrique assignent la formule: 1 acide silicotungstique–3 choline.

Détermination de la choline dans le sérum sanguin

Le sérum est déféqué par le mélange alcool–éther et la solution alcoolique est évaporée. Le résidu est repris par une solution saturée de $Ba(OH)_2$ et la saponification est poursuivie pendant 2 h. L'excès de $Ba(OH)_2$ est précipité par une solution diluée de H_2SO_4. En présence de phénolphtaléine on ajoute de la NaOH *N* jusqu'au virage et puis de l'HCl *N* goutte à goutte jusqu'à décoloration. La solution est soumise à la centrifugation. On laisse évaporer le solution aqueuse spontanément ou à une température n'excédant pas 40°. Le résidu est repris par l'alcool (filtrer ou centrifuger). La solution alcoolique est réduite à petit volume (0.2 ml). La choline est alors précipitée par la solution alcoolo-éthérée d'acide silicotungstique.

* Acide silicotungstique 50 mg; alcool 1–1.5 ml; éther *ad* 10 ml; filtrer.

** On a parfois avantage à ajouter une quantité modérée de pyridine ou de NaOH *N*. La coloration devient 2.6 × plus intense. En milieu aqueux et pour 650 mμ, une densité de 0.1 mesurée au spectrophotomètre Lumetron (diamètre du tube = 16 mm) correspond à 0.80 mg d'acide silicotungstique ou 0.73 d'"acide" silicotungstique anhydre. Par addition de pyridine ou de NaOH (précipité dissous dans 3.5 ml d'eau + 2 ml d'hydrosulfite + 0.5 ml NaOH *N* = volume final de 6 ml) une densité de 0.1 = 0.305 mg d'acide silicotungstique.

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QUANTITÉ DE CHOLINE DANS LE SÉRUM SANGUIN

Méthode au sel de Reinecke mg/ml de sérum	*Méthode à l'acide silciotungstique mg/ml de sérum*
0.10	0.11
0.61	0.64
0.21	0.22
0.11	0.10
0.20	0.21
0.34	0.35
0.56	0.54
0.24	0.23
0.86	0.90
0.19	0.21
0.40	0.42

D'autre part, des dosages effectués sur la bile et les tissus ont conduit à des résultats (trop) élevés.

Enfin, nous avons examiné l'action des divers agents de saponification et confronté les résultats obtenus en précipitant la choline par le sel de Reinecke avec ceux obtenus par précipitation à l'acide silicotungstique.

PHOSPHATIDES D'OEUF

Agent d'hydrolyse	*Préc. par Reinecke mg*	*Préc. ac. silicotungst. mg*
HNO_3 20%	3.74	2.40
NaOH *N*	3.25	2.40
$Ba(OH)_2$ sat.	3.88	3.15

PHOSPHATIDES "D'ORIGINE ANIMALE"

HNO_3 20%	2.86	2.79
NaOH *N*	1.54	2.45
$Ba(OH)_2$ sat.	2.89	2.97

De ces derniers résultats, il ressort non seulement que les agents de précipitation conduisent à des résultats qui diffèrent entre eux, mais encore qu'il y a lieu d'effectuer une sélection parmi les agents d'hydrolyse.

Nous avons d'autre part précipité de la choline par le sel de Reinecke et redissous ce précipité dans l'acétone. La solution acétonique réduite à petit volume précipite par l'acide silicotungstique. Les résultats de la réaction colorimétrique effectuée sur ce silicotungstate indiquent que la transformation du reineckate en silicotungstate est quantitative.

L'acide silicotungstique paraît en outre précipiter les phosphatides en solution éthérée sans qu'il faille attribuer cette précipitation à la présence de choline libre. D'autre part, si l'on ajoute à une solution éthérée de phosphatides de l'acétone contenant de l'acide silicotungstique, les phosphatides ne précipitent plus et inversement le précipité obtenu par addition d'acétone à une solution éthérée de phosphatides se redissout par addition d'acide silicotungstique.

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2. UNE RÉACTION COLORIMÉTRIQUE DU SEL DE REINECKE

La précipitation de la choline par le sel de Reinecke constitue un mode de dosage classique. Les nombreuses modifications apportées à la méthode portent tantôt sur le milieu de précipitation, tantôt (et surtout) sur la détermination du reineckate recueilli. Le dosage d'azote dans le précipité même, quoique bénéficiant d'un facteur de multiplication favorable (7 fois la quantité d'azote de la choline) a néammoins l'inconvénient de conduire à des résultats déficitaires lorsqu'on effectue la détermination par la méthode de Kjeldahl (fraction cholinique); d'autre part, destruction et distillation augmentent la durée de l'opération. Plusieurs auteurs[3-5,11] dosent le chrome présent dans le reineckate. La dissolution du précipité dans l'acétone et la mésure de la densité optique du reineckate comportent un mode opératoire simple et rapide[6-8]. Cependant, la détermination devient précaire ou impossible en présence de faibles quantités de reineckate. Bien plus, la coloration est instable, surtout en milieu alcalin[9]. On a essayé d'obvier à cet inconvénient en faisant des déterminations dans l'U.V.[10], mais la méthode ne peut être appliquée qu'avec des spectrophotomètres munis de dispositifs à U.V.

Dans la méthode que nous proposons, nous avons tenté d'une part d'abaisser le coefficient de solubilité et d'autre part d'effectuer un dosage colorimétrique sur des quantités extrêmement faibles de reineckate.

La précipitation

Elle s'effectue en milieu HCl *N*. On ajoute une solution saturée de sel de Reinecke dans HCl *N* à la solution à doser jusqu'à faible teinte rose. On laisse séjourner une $^1/_2$ h à la glacière et on filtre sur filtre en verre fritté.

A. Influence du volume final

Volume *ml*	*Quantité précipitée* *mg*	*Quantité trouvée* *mg*
2	1	1.07
4	1	1.04
6	1	1.01
8	1	0.98
10	1	0.98

Pour 1 mg de choline, le volume final ne doit donc pas excéder sensiblement 5 ml.

B. Influence des liquides de lavage (sur 1 mg de choline)

Lavage à l'HCl 1.2 N				*Lavage à l'éther*			
3 ×	avec 1 ml	(3 ml)	trouvé 1.07 mg	3 ×	avec 2 ml	(6 ml)	trouvé 1.01 mg
3	3	9	0.95	3	4	12	1.01
3	6	18	0.86	3	8	24	1.01

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C. Influence du liquide de lavage sur de faibles quantités de choline

Quantité de choline (mg)	*Lavage HCl 1.2 N*	*Lavage sol. éthérée*
0.90	1.14	1.01
0.66	0.76	0.76
0.45	0.44	0.41
0.23	0.09	0.25
0.12	0.00	0.09

La réaction colorimétrique

Dans le dosage classique, le reineckate est dissous dans l'acétone et de la coloration propre du sel de Reinecke on déduit la concentration en choline. Toutefois, pour de très faibles quantités de choline, la détermination devient impossible. Nous nous sommes demandés s'il ne serait pas possible de transformer le CN contenu dans la molécule en BrCN d'abord et de faire réagir celui-ci ensuite sur un dérivé pyridinique. Après de nombreux essais, nous avons adopté le mode opératoire suivant. A 1 ml de la solution acétonique de reineckate, on ajoute 2 ml d'eau de brome; on laisse réagir pendant quelques minutes, puis on ajoute 1.3 ml de solution d'arsénite de Na à 2% (veiller à ce qu'il n'y ait qu'un léger excès). Cette solution est additionnée ensuite d'1 ml de solution saturée de chlorhydrate de benzidine (solution aqueuse); 2.7 ml d'eau (volume final 10 ml) et enfin 2 ml de pyridine. La coloration se développe aussitôt. On utilise une solution standard contenant 40 μg de reineckate de choline equivalent à 13.2 μg de chlorure de choline. Toutefois, la maximum de densité optique qui, au début, se situe à 490 mμ, se déplace vers les grandes longueurs d'onde en fonction du temps. Afin d'obtenir des valeurs linéaires, il y a lieu de choisir pour chaque mesure la longueur d'onde fournissant le maximum de densité. Ainsi, pour les mesures effectuées dans les limites de 5 à 15 min, nous avons mesuré à des longueurs d'onde variant entre 490 mμ au début, à 510 mμ en fin de mesures.

Exemple. Un sérum sanguin a été soumis à l'hydrolyse. Une partie aliquote de la solution fournit après hydrolyse, un filtrat, dans lequel la choline a été précipitée par le sel de Reinecke. Le précipité recueilli a été dissous dans l'acétone et des quantités variables en ordre décroissant ont été soumises à la fois à la réaction colorimétrique directe et à la réaction au BrCN.

Réaction colorimétrique directe: il a été trouvée 2.08 mg de chlorure de choline dans 3 ml de filtrat.

Solution No.	*Quantité de filtrat mg*	*Quantité théorique mg*	*Quantité trouvée mg*
1	3	2.08	2.08
2	1.6	1.11	1.13
3	0.8	0.55	0.53
4	0.4	0.27	0.13
5	0.2	0.14	0.00

Méthode au BrCN: Les solutions acétoniques doivent être diluées au préalable pour effectuer la réaction de coloration par le BrCN.

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Longueur d'onde (mμ)	490	500	500	510	510
Temps écoulé après l'apparition de la coloration	3′	9′	12′	14′	16′
Standard 40 μg/ml D.O.	0.700	0.685	0.665	0.650	0.650
Solution acétonique No. 1 diluée au 1/16		0.630			
2 1/8		0.685			
3 1/4			0.630		
4 1/2			0.620		
5 non diluée				0.525	

Mesures et calculs fournissent les résultats suivants pour les 5 solutions.

Solution No.	*Quantité calculée mg*	*Quantité trouvée mg*
1	2.08	1.94
2	1.11	1.06
3	0.55	0.49
4	0.27	0.24
5	0.14	0.11

DÉTERMINATION DE LA CHOLINE SUR LA CHOLINE PURE

Prise d'essai mg	*Quantité trouvée par colorimétrie directe du précipité de reineckate mg*	*Quantité trouvée par la méthode au BrCN mg*
1.30	1.32	1.03
0.57	0.54	0.50
0.28	0.25	0.25
0.14	0.00	0.11
0.07	0.00	0.05
0.28	0.29	0.26
0.14	0.06	0.12
0.07	0.00	0.06

DÉTERMINATION DE LA CHOLINE DANS LE SÉRUM SANGUIN

	Quantité trouvée par colorimétrie directe mg/ml	*Quantité trouvée par la méthode au BrCN mg/ml*
	1.88	1.69
	0.52	0.42
	0.94	0.85
	1.08	0.96
	0.47	0.42
	0.21	0.21
	0.23	0.20
	0.42	0.41
Dans la bile	0.35	0.34–0.35–0.38

Des résultats de nos mesures, il ressort que la coloration que nous obtenons est près de 600 × plus intense que la coloration du reineckate de choline.

RÉSUMÉ

En solution éthérée, la choline précipite par l'acide silicotungstique; la sensibilité de la réaction atteint 1/5,000,000. La choline peut être dosée dans le sérum sanguin

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par réduction du silicotungstate obtenu; de l'intensité de la coloration bleue obtenue, on déduit la quantité de choline présente.

Le dosage par la mesure de la coloration propre du reineckate de choline devient impossible sur de très faibles quantités de précipité. La méthode que nous proposons permet l'obtention d'une réaction, dont la coloration est 600 × plus intense que celle du reineckate de choline.

SUMMARY

THE DETERMINATION OF CHOLINE IN BLOOD SERUM

In ethereal solution, choline is precipitated by silicotungstic acid. The sensitivity of this reaction is 1/5,000,000. Choline can be determined in the blood serum by reduction of the silicotungstate obtained; the amount of choline present is estimated from the intensity of the blue colour observed.

Determination by measuring the intensity of the colour of choline reineckate is not possible in the case of very small amounts of precipitate. In the method proposed by the authors use is made of a reaction which gives a colour that is 600 times as intense as that of choline reineckate.

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SERUM ENZYMES (TRANSAMINASES, PHOSPHOGLUCOMUTASE, FUMARASE) IN VIRAL HEPATITIS DURING PREDNISONE ($\triangle^1$-CORTISONE) THERAPY

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Many investigations have shown the efficacy of cortisone therapy in viral hepatitis[1-8]. The most evident results of steroid therapy found by the different authors are: rapid fall in serum bilirubin, and generally, quick regression of jaundice, remarkable improvement of appetite, acceleration of histological restoration, rapid improvement of severe hyperacute cases with clinical symptoms of hepatic coma.

Consequently cortisone therapy must be considered useful especially in severe cases with high serum bilirubin, symptoms of acute hepatic failure, or coma.

The only negative aspect of this kind of therapy is a comparatively high number of relapses (about 20% of the treated cases) after suspension[8] of the steroid administration. Hence indiscriminate application is generally considered not advisable, and the therapy should only be used in special cases.

Though the clinical aspects of cortisone therapy can be considered as well-known, the results so far acquired do not seem to explain fully the mechanism by which the therapeutical benefit is obtained.

This question becomes even more complicated by the experimental observation that mouse hepatitis virus (MHV-3)[9, 10] multiplies faster and reaches higher levels in the liver of infected mice, subjected to prednisolone ($\triangle^1$-hydrocortisone) treatment[11].

Making allowance for the differences between the viruses of human and murine hepatitis and the respective hosts and further for the specific symptoms shown by the two diseases we attempted to interpret the clinical results and to make them agree with those obtained experimentally. The fact cannot be ignored, however, that an essential condition of the animal experiment is the administration of the steroid almost from the time of the infection. This condition is practically not reproducible in clinical experiments. In fact viral hepatitis is generally suspected or diagnosed from incipient jaundice, that is to say, from a minor symptom of that disease, which is likely to appear long after the infection, probably reaching a steady maximum at a time when viral multiplication has slowed down or has perhaps become exhausted. Treatment started during such a period should be considered as late with respect to the onset of the infection.

Our previous researches[12-18] have shown in both human and experimental viral hepatitis, a remarkable increase in the activity of some of the plasma enzymes (aspartic–ketoglutaric and alanine–ketoglutaric transaminase, phosphoglucomutase, fumarase), which are normally either very low or entirely absent. Other enzymic activities appear in the blood plasma only in murine hepatitis while they are absent in the infected human plasma (alcohol dehydrogenase, glutamic dehydrogenase[19]). These results have been interpreted as the passage into the bloodstream of enzymic

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proteins from necrotic foci of the parenchyma produced by viral multiplication in the liver.

In our opinion, the observation of these enzymic variations may provide more objective information concerning the course of the disease than do serum bilirubin determinations or serum lability tests. In fact, the increased enzymic activities of the plasma indicate the dynamic course of the essential lesion of viral hepatitis: the necrosis of the liver parenchyma.

These considerations led us to studying the course of these enzymic activities in patients with acute viral hepatitis, during treatment with prednisone ($\triangle^1$-cortisone).

This investigation was expected to clarify the mechanism of this treatment, besides giving some information concerning its influence on the course of the disease.

EXPERIMENTAL AND RESULTS

14 male subjects suffering from viral hepatitis in the active phase were observed.

In some of these cases (Nos. 2, 5, 6, 7, 9, 10, 13), treatment was started only a few days after jaundice had appeared; in others, (Nos. 1, 3, 4, 8, 11, 12, 14) it began when jaundice had lasted for some weeks. This second group comprised rather severe cases, in which the course of the disease lasted longer than usual.

Treatment was carried out with prednisone* with an initial dose of 40 mg per day, that was later reduced to 30 and 20 mg. In two particularly grave cases, the initial doses were of 100 mg per day.

Enzyme determinations were made in the serum using the methods reported by us previously[12, 18]. At the same time serum bilirubin was determined and tests of serum colloidal lability (TAKATA-ARA, HANGER, MCLAGAN) were performed.

The results are reported in the graphs 1 to 14.

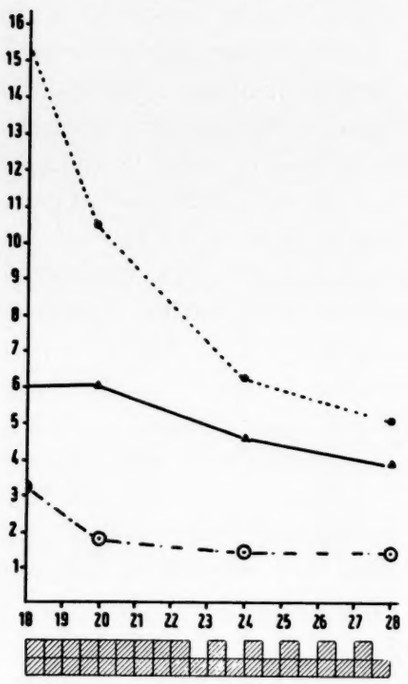

Fig. 1.

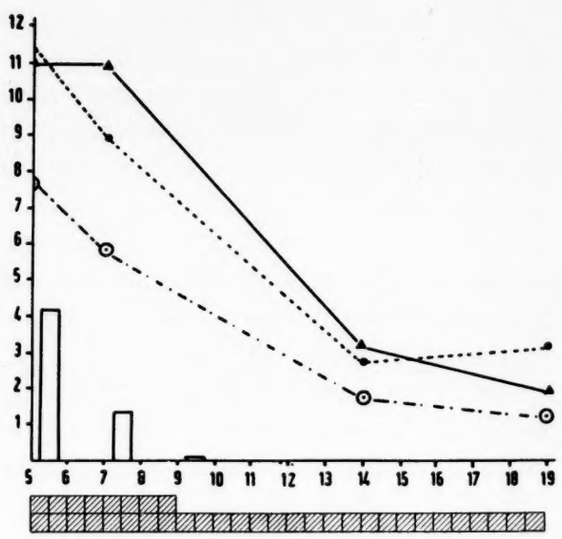

Fig. 2.

* Deltacortene ($\triangle^1$-cortisone), kindly supplied by Lepetit S.A.

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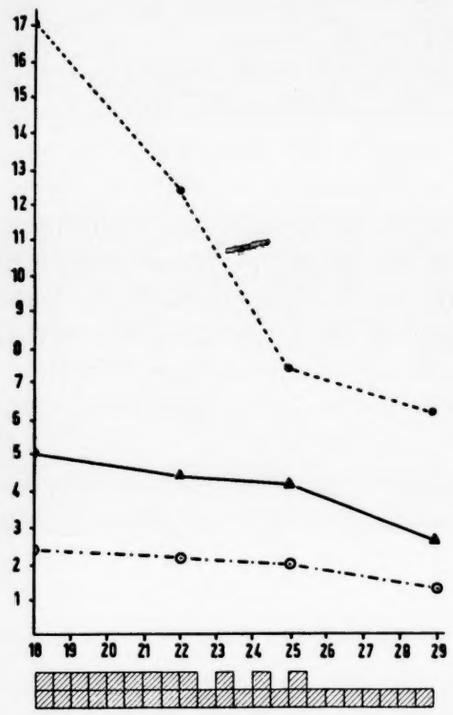
Symbols used in all Figs.:

Abscissa = days from the onset of icterus
Ordinate = values of enzyme activities and of serum bilirubin
Serum bilirubin (mg%/ml)
Alanine–ketoglutaric transaminase activity
Aspartic–ketoglutaric transaminase activity
(μmoles of ketoacid, pyruvate or oxaloacetate, formed by 1 ml of serum in 15 min at 37°)
Phosphoglucomutase activity (μmoles of G–1–P converted to G–6–P by 1 ml of serum in 60 min at 30°)
Fumarase activity (units/ml of serum)
Prednisone 10 mg



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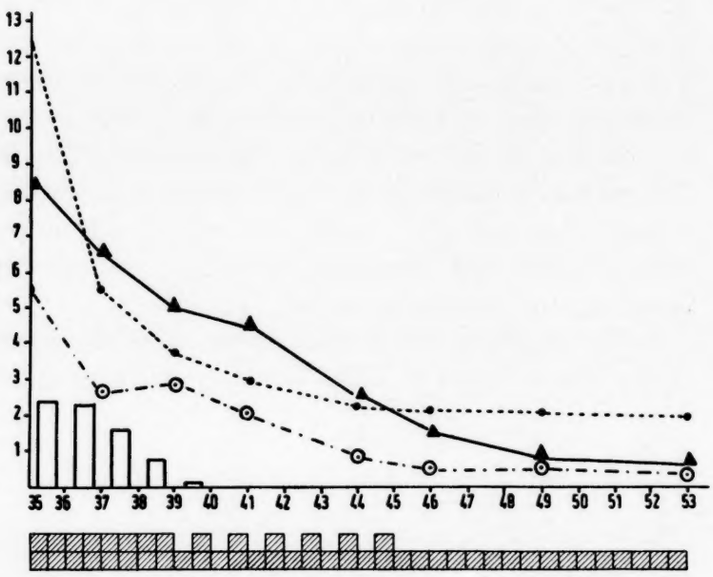


Fig. 4.

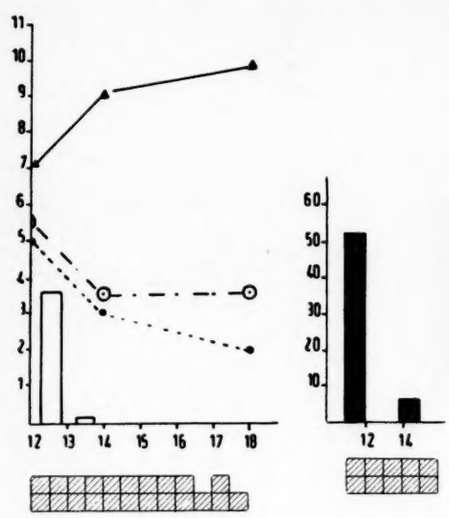


Fig. 5.

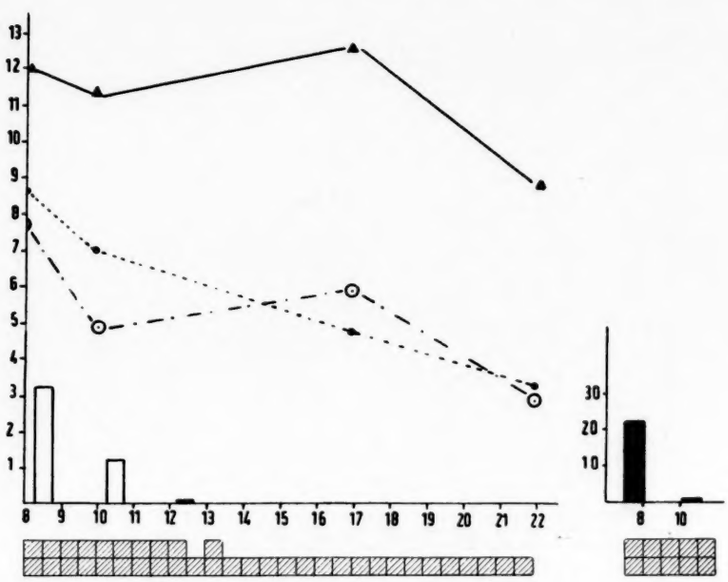


Fig. 6.

DISCUSSION

A decline of serum enzyme activities and of serum bilirubin was observed in most of the 14 cases investigated. This was prompt in some cases (Nos. 4, 7, 8, 10, 11, 12, 13), more gradual in others (Nos. 1, 2, 14). In the remainder, there were either very slow and less marked decreases (Nos. 3, 6, 9), or some discordance between the various enzyme activities (Nos. 5, 9). Serum bilirubin generally decreased to the same degree as transaminase activity (with the exception of cases Nos. 6 and 11).

All the enzyme activities studied showed approximately the same pattern. Nevertheless, some of these (fumarase, phosphoglucomutase), where present, seemed to be in general more responsive, because they promptly disappeared, or dropped to a low level.

The question arises therefore, whether the enzymic modifications observed were an effect of the prednisone therapy, or merely due to a chronological coincidence. However, in order to answer this question, we should have to follow a corresponding number of patients receiving no steroid therapy, and to compare at regular intervals and systematically the values of the enzyme activities in the sera of the 2 groups. However, it is obviously difficult to choose two comparable groups of patients as regards the phase and general characteristics of the disease, since viral hepatitis is a disease which often shows wide spontaneous variability in evolution and severity. Nevertheless, in our opinion, prednisone therapy induced the reduction and the disappearance of enzymic activities and of serum bilirubin. This conclusion is based on the following facts.

Modifications have been observed both in cases where jaundice had appeared only a few days before (Nos. 2, 5, 6, 7, 9, 10, 13) and the disease presented on the whole

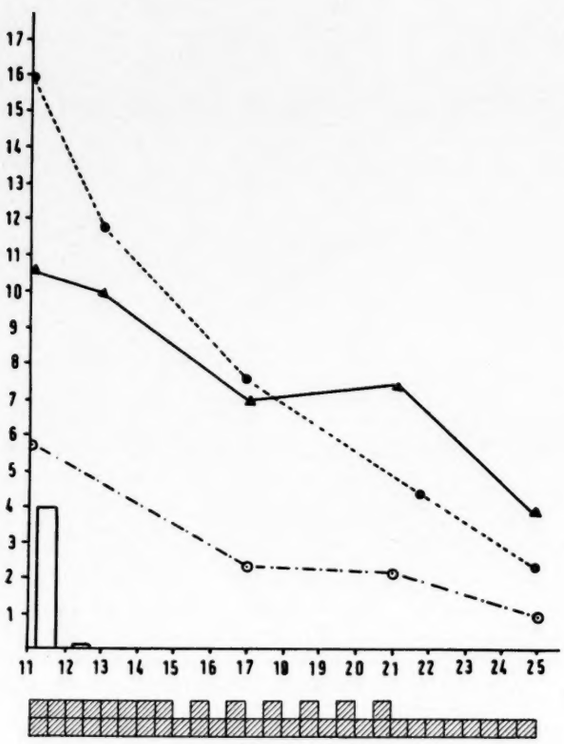
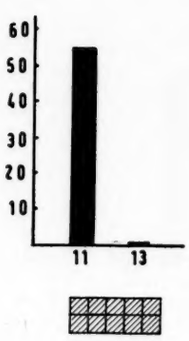


Fig. 7.



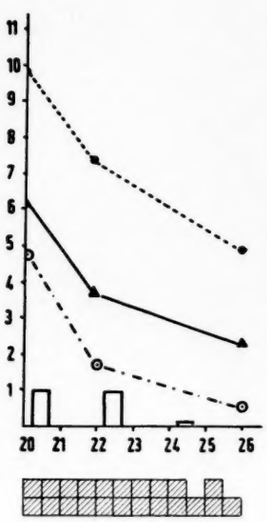


Fig. 8.

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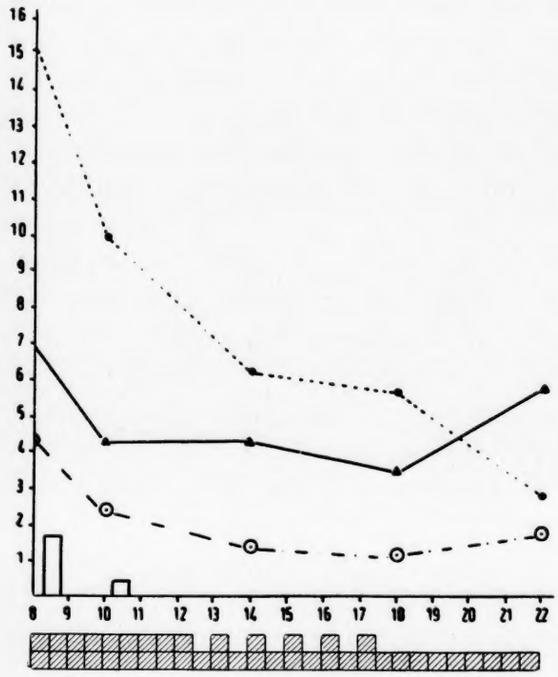

Fig. 9.

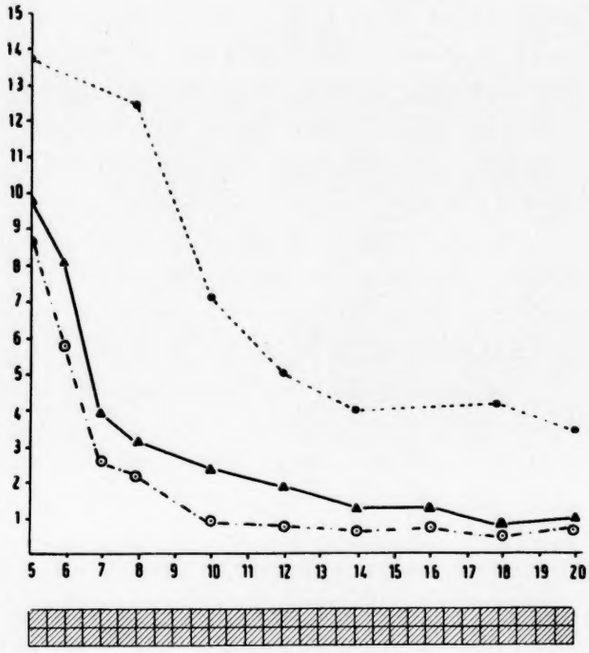

Fig. 10.

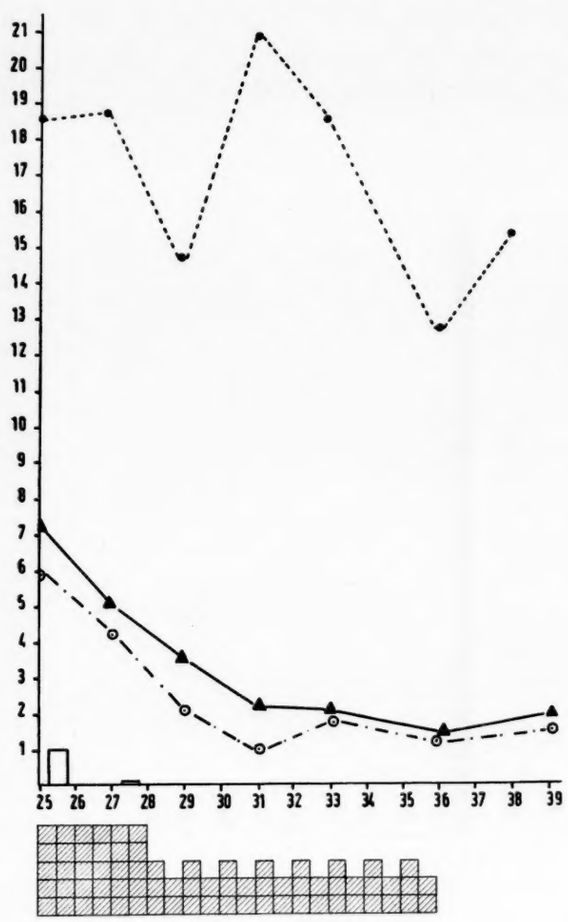

Fig. 11.

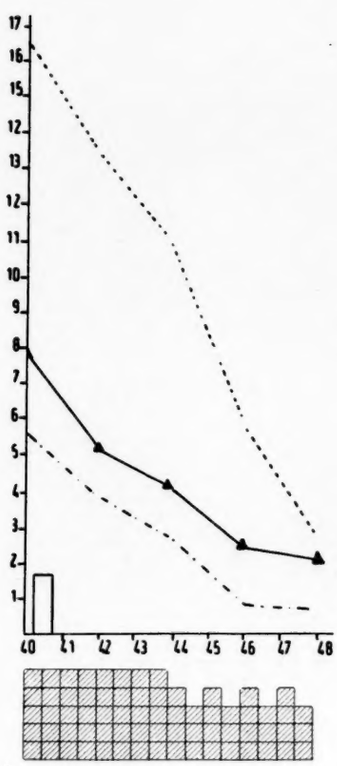

Fig. 12.

its usual benign aspect, and, in cases where jaundice had lasted for some weeks or over a month (Nos. 1, 3, 4, 8, 11, 12, 14). In these cases the disease showed remarkable severity from its onset and did not regress spontaneously but even so a rapid recovery and a prompt decline of enzymic values followed prednisone therapy.

The fall in some or all enzyme activities was really dramatic in many cases: transaminase activity dropped in a few days, fumarase and phosphoglucomutase in only 24 hours.

In the second group of patients, it would be difficult to interpret this phenomenon as a spontaneous evolution of the disease which, in the phase studied by us, had the characteristics of a subacute process with no appreciable probability of a prompt recovery.

Other arguments confirming our assumption derive from the graphs of some cases (Nos. 6, 11) which show a significant decrease in the values of transaminase activity without serum bilirubin being sensibly influenced. These differences would be almost incomprehensible in a spontaneous recovery.

These results lead one to consider whether these plasma phenomena, resulting from different pathogenetic factors, may not be variously influenced by this treatment, as the variations in serum bilirubin and in enzyme activity are not always simultaneous—even though their pattern is generally similar.

Serum bilirubin may be influenced in different ways by prednisone therapy, *viz.* by:

a) Removal of cholostatic components through elimination of pericanalicular inflammation, due to the antiphlogistic action of prednisone (see [7]).
b) Diminution of biligenesis as demonstrated in obstructive jaundice [20].

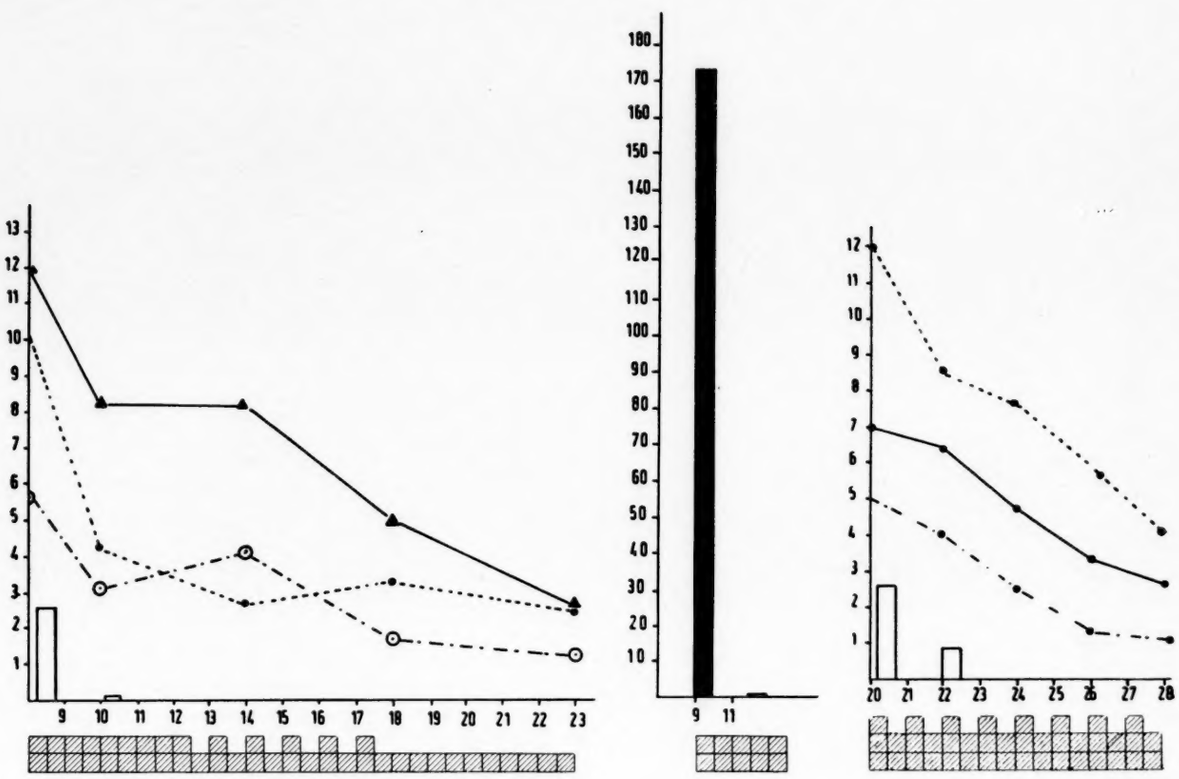

Fig. 13.

Fig. 14.

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c) Improvement of the functions of the hepatic cells connected with the metabolism and excretion of bilirubin.

No connection has been established between the decrease of enzymic activities and the course of the serum colloidal lability so that the rapid decrease of plasma enzyme activities produced by prednisone administration seems to be independent of these facts.

We feel that the appearance of, or the increase in activity of some of the plasma enzymes which occurs in viral hepatitis and in other conditions of human and experimental pathology is strictly connected with cellular necrosis.

Hypothetically, the reduction in the values of the enzyme activities of plasma could be attributed to a generic antinecrotic action of prednisone, but, this interpretation has not been confirmed by our researches which showed that experimental liver necrosis in mice due to CCl_4 is not appreciably modified by prednisone[23].

However it can be assumed that in viral hepatitis prednisone therapy can quickly stop the necrotic process through some indirect mechanism (antiphlogistic action, improvement of metabolism of hepatic cells, etc.[7]

Anyway, whatever interpretation is given to our observations, it is certain that the study of plasma enzyme activities in the course of therapy represents a new approach reflecting more closely the real anatomic–clinical evolution of the disease than the study of other phenomena (jaundice, serum colloidal lability) which appear later. In fact, the variations of the enzymic activities of plasma seem to reflect more clearly the course of the biochemical phenomena occurring in the hepatic tissue.

The present investigations are the first attempt to clarify the mechanism of steroid treatment in viral hepatitis and, we wish to emphasize that in all cases, the course of the disease was quickly and favourably influenced, and no relapses occurred. We presume therefore, that prednisone therapy can be used in every case of viral hepatitis though theoretically, those cases should be excluded in which persistence of active viral multiplication in the hepatic tissues is suspected, and in which, therefore, relapses may occur.

However such cases are not likely to be very frequent considering that infection occurs several weeks before the appearance of jaundice[21, 22] and that when submitted to medical observation, all cases are in the icteric phase, that is in a late phase.

Probably, in that phase, the infectious process is declining—as a consequence of the onset of immunity and the relative exhaustion of receptive cells—and the disease is on the point of being transformed from infectious into a "metabolic" one as becomes evident in the cases developing into postnecrotic cirrhosis.

SUMMARY

The pattern of some enzyme activities (transaminases, phosphoglucomutase, fumarase) has been investigated in cases of virus hepatitis during treatment with prednisone ($\triangle^1$-cortisone).

This steroid therapy seems to suppress readily some serum enzyme activities and to diminish others promptly.

The theoretical and pathogenetic background of this observation is briefly discussed.

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ZUR BESTIMMUNG DER SERUMLIPASE
I. BESTIMMUNG MIT PHENYLLAURAT ALS SUBSTRAT

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Obwohl zur Bestimmung von Serumlipase eine Reihe von Methoden angegeben wurde, bestehen immer noch Schwierigkeiten bei der exakten routinemässigen Kontrolle einer grösseren Anzahl von Serumproben in der Klinik. Neben der Messung der Abnahme der Oberflächenspannung von RONA UND MICHAELIS[1] werden vor allem titrimetrische Verfahren benutzt, die die durch die Lipasewirkung freiwerdende Säure durch direkte Titration mit Lauge gegen Phenolphthalein als Indikator oder an der Glaselektrode ermitteln (CHERRY UND CRANDALL[2], HEINKEL[3] u.a.). AMMON[4, 5] benutzte ein manometrisches Verfahren, bei dem die durch die Lipasewirkung freiwerdende Säure CO_2 aus der als Verdünnungsmedium fungierenden Ringerlösung freisetzte.

Alle diese Methoden haben den Nachteil, dass sich das pH der Lösung während der Inkubation durch die freiwerdende Säure verändert und weiter, dass die Reaktion in einem biphasischen System (Emulsion) vor sich geht. Dadurch ist die Abhängigkeit der Substratspaltung von der Enzymmenge nicht linear, und man muss durch Eichkurven ermittelte Korrekturen benutzen. Den ersten Nachteil kann man dadurch ausschalten, dass anstelle der freiwerdenden Säure die alkoholische Komponente der Fettsäureester bestimmt und das pH während der Inkubation durch eine Pufferlösung konstant gehalten wird*. So benutzten PURR[8] und RUPPERT[9] Phenolphthalein-dibuttersäureester. Das freigesetzte Phenolphthalein konnte direkt in alkalischer Lösung fotometrisch ausgewertet werden. Bei der Kontrolle dieser Methode mussten wir jedoch feststellen, dass auch hier das Verhältnis des abgespaltenen Phenolphthaleins nicht proportional der Enzymmenge ist, sondern dass zwischen der Enzymmenge und dem freigesetzten Phenolphthalein eine hyperbolische Funktion besteht. Als Ursache für den parabolischen Zusammenhang nehmen wir eine unterschiedliche Spaltungsgeschwindigkeit der beiden Estergruppen am Phenolphthalein-dibuttersäureester an.

Auf der Suche nach einem besseren Substrat wandten wir uns deshalb Fettsäureestern von einwertigen Fettsäuren mit einwertigen Phenolen zu. SELIGMAN UND

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* Zu wissenschaftlichen Untersuchungen kann man enzymatische Reaktionen bei konstantem pH in ungepufferter Lösung mit dem pH-stat durchführen. Hierbei wird das pH der Substratlösung während der Inkubation dauernd mit Indikatoren oder mit einer Glaselektrode gemessen und aus einer Bürette Lauge zur Konstanthaltung des pH zugegeben. Am Ende der Inkubationszeit kann man dann sofort die Gesamtmenge der verbrauchten Lauge und die ml/Zeiteinheit an einem Kontrollstreifen ablesen (KNAFFL-LENZ[6], WHITNAH[7]).

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NACHLASS[10] benutzten bereits β-Naphthyllaurat. Das fermentativ freigesetzte β-Naphthol wurde mit Tetraazo-di-*o*-anisidin zu einem roten Farbstoff gekuppelt und dieser mit Äthylazetat ausgeschüttelt. Zur Trennung der Emulsion wird die Mischung zentrifugiert. Hierzu benötigt man Zentrifugengläser mit Schliffstopfen, die unserer Erfahrung nach sehr leicht brechen. Andererseits wird die Bestimmung durch Verdunstungsfehler erschwert und der Fehler erhöht.

Aus diesem Grunde wurden von uns Laurinsäureester des Nitrophenols und Phenols für die Lipasebestimmung erprobt. Die Methode mit Phenyllaurat als Substrat erwies sich für den klinischen Routinebetrieb am geeignetsten. Sie wird deshalb im folgenden beschrieben:

Prinzip: Phenyllaurat wird im Gegenwart von Cholat spezifisch durch Serumlipase gespalten. Das proportional zur Enzymmenge freigesetzte Phenol bildet mit Folinreagens in alkalischer Lösung Wolframblau.

METHODE

Reagenzien

1. *Phenyllaurat.* 20 g Laurinsäure wurden in 25 ml Thionylchlorid (Kp. 75°) gelöst. Die Mischung wurde 10 min bei 70° gehalten und danach das überschüssige Thionylchlorid im Vakuum abdestilliert. Zur heissen Lösung wurden 9.5 g Phenol und 10 ml trockenes Pyridin gegeben. Die Mischung wurde 15 min bei 80° gehalten. Nach dem Abkühlen wurde das Ganze mit 150 ml Azeton versetzt und zur Lösung 10 g Aktivkohle gegeben. Das schwach gelbliche Filtrat wurde zu einem Eis–Wasser-Gemisch gegeben und die ausfallenden gelblichen Kristalle abfiltriert. Die Kristalle wurden in Petroläther gelöst und die Lösung von Pyridinresten durch dreimaliges Ausschütteln mit destilliertem Wasser entfernt. Anschliessend destillierten wir den Petroläther im Vakuum ab und fällten erneut durch Zugiessen in ein Eis–Wasser-Gemisch. Trocknen der Kristalle im Vakuum über Kalziumchlorid. Ausbeute rein *ca.* 20 g, Smp. 18° (unkorrigiert). Im Kühlschrank aufbewahren. *Löse* 250 mg Phenyllaurat in 25 ml Azeton.

2. *Veronal–Azetat-Puffer nach Michaelis, pH* 7.4. 9.714 g Natriumazetat ($CH_3COONa \cdot 3\,H_2O$) und 14.714 g Veronalnatrium werden in CO_2-freiem Wasser zu 500 ml gelöst, 500 ml 0.1 *N* HCl und 1300 ml CO_2-freies Wasser hinzugegeben.

3. *Natriumcholatlösung* (20.0 g/100 *ml*).

4. *Substratlösung* (frisch bereiten). Mische 10 ml Pufferlösung (pH 7.4) mit 35 ml Wasser und gib aus einer Pipette mit feiner Ausflussöffnung, die in die Flüssigkeit eintaucht, 10 ml der Phenyllauratlösung unter Umschwenken hinzu. Es entsteht eine kolloidale Lösung.

5. *Reagens nach Folin.* Löse 100 g Natriumwolframat ($Na_2WO \cdot 2\,H_2O$) und 25 g Natriummolybdat ($Na_2MoO_4 \cdot 2H_2O$) in 700 ml destilliertem Wasser auf. Gib 50 ml Phosphorsäure (85 g/100 ml) und 100 ml Salzsäure ($d = 1.2$) hinzu und koche das Ganze 10 Stunden am Rückflusskühler. Darauf werden 150 g Lithiumsulfat (Li_2SO_4), in 50 ml Wasser gelöst, hinzugegeben und weiter 5–10 Tropfen flüssiges Brom. Der Überschuss des Broms wird durch 15 Minuten-langes Kochen entfernt und nach dem Abkühlen auf 1 l mit destilliertem Wasser aufgefüllt. Kühl in dunkler Flasche aufbewahren. Zum Gebrauch wird diese Stammlösung 1 : 2 mit destilliertem Wasser verdünnt.

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6. *Natriumkarbonatlösung* (6 *g*/100 *ml*).

7. *Phenolstandard.*

(a) Stammlösung (*ca.* 1 mg/ml). Gib ungefähr 1.2 g Phenol p.a. in einen 1 l-Messkolben und löse in ungefähr 200 ml 0.1 *N* HCl auf. Fülle mit 0.1 *N* HCl bis zur Marke auf.

(b) Verdünnter Phenolstandard (0.05 mg/ml). Gib 5 ml der Phenolstammlösung in eine 100 ml-Flasche und fülle mit destilliertem Wasser auf. Im Kühlschrank 1 Monat lang haltbar.

(c) Eichung. 25 ml der Phenolstammlösung werden in einen 250 ml-Erlenmeyer-Schliffkolben gegeben, 50 ml einer 0.1 *N*-Natriumhydroxydlösung hinzugegeben und die Mischung auf 65° erwärmt. Darauf werden 25 ml 0.1 *N* Jodlösung hinzugegeben und gemischt. Nach Verschliessen des Kolbens mit einem Schliffstopfen lasse man die Mischung 45 min bei Raumtemperatur stehen. Darauf werden 5 ml konz. Salzsäure hinzugegeben und der Überschuss an Jod mit 0.1 *N* Natriumthiosulfat unter Verwendung von 1 ml Stärke-Lösung (1 g/100 ml) als Indikator zurücktitriert.

Berechnung: mg Phenol in 25 ml = 1.568 × (ml 0.1 *N* Jodlösung — ml 0.1 *N* Natriumthiosulfat). Werden mehr als 25 mg Phenol in 25 ml gefunden, wird die Stammlösung mit 0.1 *N* Salzsäure zu dieser Konzentration verdünnt.

Durchführung

0.2 ml Plasma oder Serum werden mit 5 ml Substratmischung und 0.5 ml Natriumcholatlösung versetzt. Nach dem Umschütteln lässt man die Mischung genau 30 min bei 37° inkubieren. Darauf werden 3 ml Folinreagens hinzugegeben und nach einer Minute zentrifugiert (10 min 2000 *g*). 2 ml des klaren Zentrifugates werden mit 8 ml Natriumkarbonatlösung versetzt, umgeschüttelt und nach 20 min die Intensität der sich entwickelnden blauen Farbe fotometrisch gemessen (Rotfilter RG 2. Lange-Photometer, Adsorptionsmax. 650 bis 700 mμ).

Zur Bestimmung des Leerwertes werden 5 ml Substratmischung mit 0.5 ml Cholatlösung vermischt und nach 30 min Inkubation bei 37° 0.2 ml Plasma zugegeben. Darauf wird sofort mit Folinreagens gefällt und weiter wie oben verfahren.

Standardwert

1 ml des verdünnten Phenolstandards wird mit 4.7 ml Wasser und 3 ml Folinreagens versetzt. Nach dem Umschütteln werden 2 ml dieser Mischung mit 8 ml Natriumkarbonatlösung gemischt und die sich entwickelnde Farbe wie oben gemessen.

ERGEBNISSE

(a) In Fig. 1 ist die Spaltung von Phenyllaurat in Abhängigkeit der Enzymmenge dargestellt. Es wurden hierbei zu einer gleichbleibenden Serummenge (0.2 ml) steigende Mengen Pankreasextrakt gegeben. Es zeigt sich, dass der Ester direkt proportional zur Enzymmenge gespalten wird. Erst wenn mehr als 50% des Substrates hydrolysiert sind, konnten wir eine Abweichung von dieser Proportionalität feststellen.

(b) Fig. 2 zeigt die Abhängigkeit der Lipaseaktivität von der Cholatkonzentration des Inkubationsansatzes. Mit zunehmender Cholatkonzentration steigt die Aktivität

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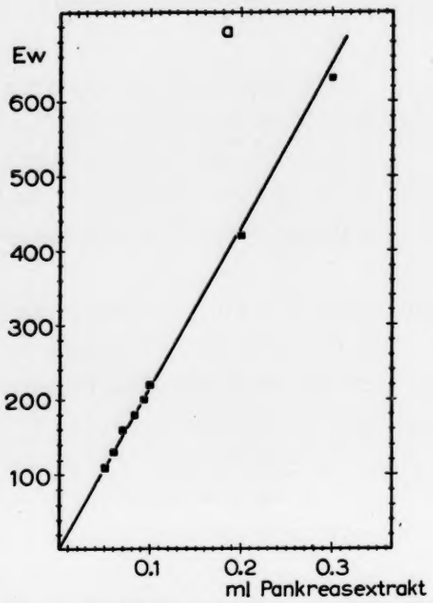

Fig. 1a. Abhängigkeit der Substratspaltung von der Enzymmenge. Die im Gemisch mit Cholat 100 mg, Serum 0.2 ml steigende Pankreasextraktkonzentration wurde gegen Extinktion aufgetragen. Wahre Extinktion (*Ew*) = abgelesene Extinktion — Leerwert — Serumwert von 0.2 ml. Pankreasextrakt: Pankreatin Schering 0.1 g/100 ml, filtriert.

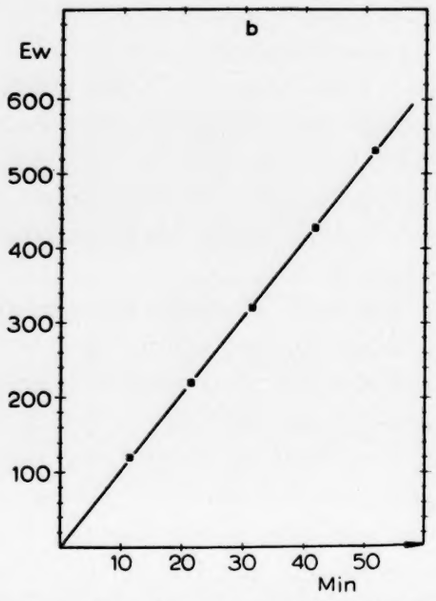

Fig. 1b. Zeitabhängigkeit der Substratspaltung durch Lipase.

der Lipase an. Bei 100 mg Cholat/Ansatz wird das Maximum der Aktivität erreicht. Oberhalb 200 mg tritt Hemmung auf.

(c) Fig. 3 zeigt die pH-Abhängigkeit der Substratspaltung durch Serumlipase in Veronal–Azetat-Puffer nach MICHAELIS.

(d) Fehler der Methode. 19 Einzelbestimmungen, gemessen mit dem Lange-Photometer, Filter RG 2, Max. 650–700 mμ (Tabelle 1).

TABELLE I

1	2	3	4	5	6	7	8	9	10
0.447	0.443	0.448	0.450	0.445	0.447	0.445	0.448	0.450	0.446

11	12	13	14	15	16	17	18	19
0.449	0.447	0.449	0.449	0.451	0.443	0.448	0.442	0.451

$$M = 0.447;\ fm = \sqrt{\frac{f^2}{n-1}} = \sqrt{\frac{131}{18}} = 2.7 \cdot 10^{-3};\ M = 0.447 \pm 0.0027;\ \frac{fm}{M} = 0.6\%$$

DISKUSSION

Bei der Lipasebestimmung mit Phenolphthalein-dibutyrat nach PURR[8], die von RUPPERT[9] in die Klinik eingeführt wurde, besteht zwischen der abgespaltenen Menge Phenolphthalein und der Enzymkonzentration ein hyperbolischer Zusammenhang. Deshalb ist es *nicht zulässig*, ohne Benutzung einer hyperbolischen Eichkurve die

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Extinktion bzw. das dieser Extinktion entsprechende Phenolphthalein direkt als Mass für die Enzymaktivität zu nehmen. Man muss vielmehr zur Auswertung dieser Methode in analoger Weise wie HUGGINS und TALALAY[11] vorgehen. Sie stellten bei der Untersuchung der Phosphatase mit Phenolphthalein-diphosphorsäureester ebenfalls einen hyperbolischen Zusammenhang zwischen der abgespaltenen Phenolphthaleinmenge und der Enzymkonzentration fest und ermittelten die Phosphataseeinheiten der zu untersuchenden Proben aus einer sich aus diesem Zusammenhang ergebenden hyperbolischen Eichkurve. Mit steigender Enzymmenge nimmt hierbei die Ablesegenauigkeit ab. Unserer Meinung nach sind daher für die Bestimmung der Lipase grundsätzlich Ester aus einwertigen Alkoholen und einwertigen Fettsäuren vorzuziehen.

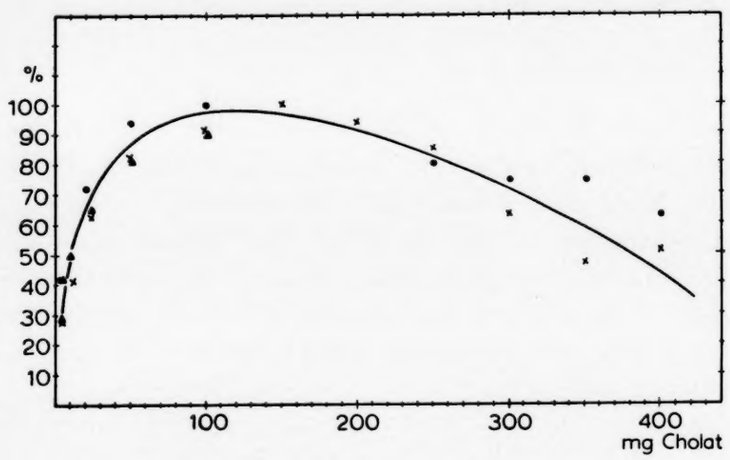

Fig. 2. Abhängigkeit der Lipaseaktivität von der Cholatkonzentration im Inkubationsansatz. % der Maximalaktivität gegen mg Cholat/Ansatz.

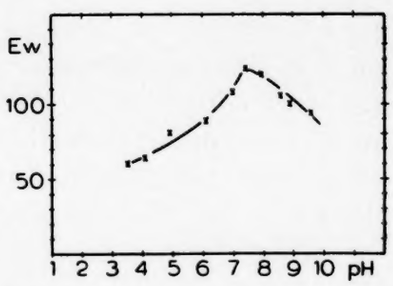

Fig. 3. pH-Abhängigkeit der Spaltung von Phenyllaurat durch Serumlipase. Veronal–Azetat-Puffer nach MICHAELIS.

Aus Nitrophenyllaurat durch die Lipasewirkung freigesetztes Nitrophenol konnten wir nach Enteiweissen des Inkubationsansatzes mit Quecksilbermischung nach HINSBERG[12] in der alkalischen Lösung direkt messen (unveröff. Versuche). Jedoch ist die fotometrische Auswertung der gelben Farbe mit visuellen und fotoelektrischen Routinephotometern schwierig. Die benötigten blauvioletten Filter haben eine so geringe Durchlässigkeit, dass die Empfindlichkeit der Augen- bzw. Fotozellen für die Messung nicht ausreicht. Wir benutzen deshalb Phenyllaurat als Substrat. Hierbei gibt das durch die Lipasewirkung freigesetzte Phenol mit Folinreagens eine blaue Farbe. Gleichzeitig wird das Eiweiss des Inkubationsgemisches gefällt, und man erhält eine leicht zu fotometrierende, klare, blaue Lösung.

Das Substrat ist in dem Inkubationsansatz hoch kolloidal verteilt und verhält sich gegenüber dem Enzym wie eine echt gelöste Verbindung. Die Substratspaltung bzw. die Farbentwicklung folgt direkt proportional der Enzymkonzentration und bei gleicher Enzymkonzentration direkt proportional der Zeit (Fig. 1). Ein Schütteln des Ansatzes während der Inkubation erübrigt sich.

Das pH-Optimum in Veronal–Azetat nach MICHAELIS bestimmten wir für die Serumlipase des Menschen zu pH 7.4. Da bei diesem pH die Aktivität der Lipase längere Zeit erhalten bleibt, wählten wir diesen Puffer für unsere Methode. Die Verwendung von Borax–Phosphatpuffer nach RUPPERT[9], in welchem die Serum-

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lipase ein pH-Optimum von *ca.* 10 aufweist (unveröff. Versuche), ist unzweckmässig. Bei der von RUPPERT angewandten Inkubationsdauer bei pH 9 von mehr als 20 Stunden wird die Serumlipase weitgehend zerstört. Nur die Leberesterase bleibt erhalten und kann gemessen werden (vgl. YAMAMOTO[13]).

Cholat dient zur Aktivierung der Lipase. Gleichzeitig werden die anderen Esterasen des Serums gehemmt (NISHART[14]). 100 mg Cholat im Ansatz bewirken eine Aktivierung um 300% (Fig. 2). Aus der Aktivierungskurve geht ausserdem deutlich hervor, dass das Substrat Phenyllaurat durch Esterasen des Serums in der angegebenen Methodik fast nicht gespalten wird. Die angegebene Methode ist daher als spezifisch für Serumlipase anzusehen.

Der Fehler der Einzelmessung ist bei exaktester Arbeit 0.6 (Tabelle I). Bei Routinebestimmungen steigt er bis zu 1.2% an.

ZUSAMMENFASSUNG

Es wird eine exakte und spezifische Methode zur Bestimmung der Serumlipase angegeben. Als Substrat wird Phenyllaurat verwandt. Die enzymatische Spaltung des Substrats Phenyllaurat erfolgt direkt proportional zur Enzymkonzentration und zur Zeit. Das pH-Optimum für die Serumlipase des Menschen liegt bei Verwendung von Azetat-Puffer nach MICHAELIS mit einem Cholatzusatz von 50 mg/Ansatz und unter Verwendung von Phenyllaurat als Substrat bei pH 7.4. Die Aktivierung der Serumlipase durch einen Zusatz von Natriumcholat (100 mg/Probe) liegt bei 300%. Der Fehler der Einzelmessung beträgt 0.6–1.2%.

SUMMARY

DETERMINATION OF SERUM LIPASE WITH PHENYL LAURATE AS SUBSTRATE

An accurate and specific method is described for determining lipase in serum, using phenyl laurate as substrate. Enzymic splitting of the phenyl laurate substrate is directly proportional to the enzyme concentration and to the time of incubation. With phenyl laurate as substrate the pH optimum for human serum lipase is 7.4 when an acetate buffer as described by MICHAELIS is used and 50 mg cholate is added to each sample. Addition of sodium cholate (100 mg per sample) activates serum lipase to an extent of 300%. The experimental error is 0.6–1.2%.

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AN IMPROVED METHOD FOR THE ESTIMATION OF CHOLINESTERASE ACTIVITY IN SERUM

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Measurement of the serum cholinesterase activity (Ch.E.A.) has become a popular examination in the every day laboratory and its value in various conditions, such as in liver and biliary diseases[1], in malnutrition[2] in fluorophosphate ester poisoning[3] and in anesthesiology[4] has been described by several authors. MICHEL's[5] method although valuable, is not suitable for everyday routine use since it requires the use of a pH meter and is too time-consuming for the performance of a large number of tests. Methods developed more recently[6, 7] are based on measurement of the decreasing optical absorption of the phenol red indicator due to the acidity produced by the splitting of acetyl choline by the enzyme. These methods have been adopted by most clinical laboratories because of their simplicity and the fact that they can be performed in relatively large numbers at the same time. Our method is based on similar principles, but its advantages are that by substituting the phenol red indicator with *m*-nitrophenol[8], the "protein error", which is caused by the selective binding of the phenol red to different protein fractions, is avoided. Moreover, the phenol red indicator changes from red to orange, and to yellow during incubation, thereby causing a shift of the wavelength in which its transmission is measured. The *m*-nitrophenol indicator has a one-colour reaction, and covers the same pH range as the phenol-red without deviations in the wavelength, thereby giving accurate results.

In order to be able to perform a large number of tests at the same time, it was found necessary to inactivate an amount of serum equal to that used in the test instead of "zero timing" it.

METHOD

Reagents

1. *Buffer. (a).* Dissolve 6.65 g of anhydrous disodium phosphate (Na_2HPO_4) and of 0.43 g of monopotassium phosphate (KH_2PO_4) in about 200 ml of distilled water. *(b).* Dissolve 0.30 g of *m*-nitrophenol in about 200 ml of distilled water (might be heated slightly). Mix *a*, and *b*, adjust the pH to 7.8 by the addition of *N*/10 NaOH, and make up the volume to 1000 ml with distilled water.

2. *Acetylcholinechloride* 15% (keep in the refrigerator).

3. 0.9% *NaCl.*

4. *Acetic acid* 0.1 *N.*

Procedure

Put 0.1 ml NaCl solution into each of two test tubes (B and T), and add 0.1 ml of serum. Inactivate cholinesterase in one of the tubes (B) by placing it in a water

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bath at 60° for 3 min, allow to cool, and add 2.5 ml of buffer and 0.1 ml of acetylcholinechloride solution to both tubes. Incubate at 25° for 30 min, then read in the colorimeter setting the zero with distilled water using blue filter. Deduct value of test (T) from the blank (B) and obtain result of cholinesterase units from the calibration chart.

Calibration

Prepare 11 test tubes. Put into each tube 2.5 ml of buffer, 0.1 ml of inactivated pooled serum (avoid the use of hemolysed, icteric or turbid sera) and 0.1 ml of acetylcholinechloride. To the first tube add 0.1 ml of distilled water (B) and into the others 0.1 ml of acetic acid in increasing concentrations from 0.01 *N* to 0.10 *N* with increments of 0.01 *N* from tube to tube (S_1–S_{10}). Read in the colorimeter, setting zero with distilled water, using the blue filter. Deduct all *S* values from the reading of B and prepare a calibration curve (Fig. 1) on millimetric paper, each increment of 0.01 *N* acetic acid representing 10 cholinesterase units[7]. For every new batch of reagents a new calibration curve has to be prepared. A simple method for the preparation of the calibration standards is presented in Table I.

TABLE I

PREPARATION OF STANDARD SOLUTIONS FOR PLOTTING A CALIBRATION

No. of test tubes	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0.1 *N* Acetic acid, ml	0	1	2	3	4	5	6	7	8	9	10
Distilled water, ml	10	9	8	7	6	5	4	3	2	1	0
Ch. E. A. units	0	10	20	30	40	50	60	70	80	90	100

In case of results higher then 100 units a dilution of the serum 1 : 1 has to be made and the test repeated

DISCUSSION

We were interested to know whether *m*-nitrophenol has any inhibitory effect on cholinesterase activity, so we incubated several samples of serum in a buffer–substrate mixture which did not contain indicator. Since we obtained identical results

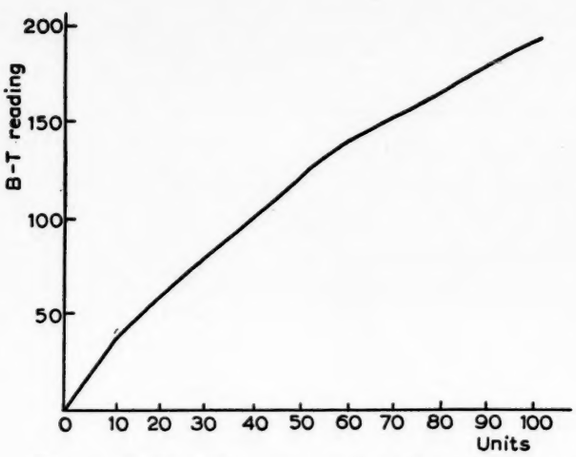

Fig. 1. Calibration curve of cholinesterase activity

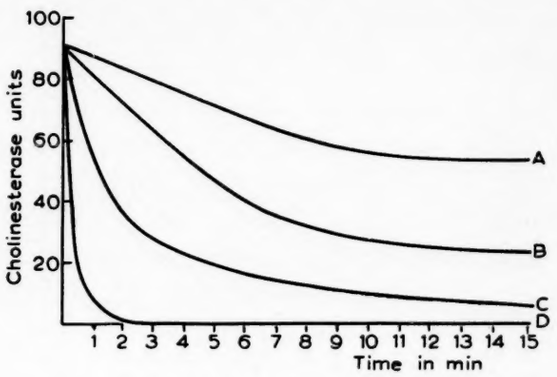

Fig. 2. Rate of inhibition of cholinesterase activity by exposing serum to various temperatures. Curve A = 40°; Curve B = 50°; Curve C = 56°; Curve D = 60°.

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with the controls we concluded that there are no inhibitory effects. We also found that the alkaline phosphatase, an enzyme much more susceptible to chemical agents, is not inhibited by nitrophenol. We found cholinesterase to be an enzyme highly resistant to most enzyme inhibitors and sodium fluoride, sodium azide, periodate, mercuric oxycyanate, arsenate, lead acetate, aniline citrate, quinine, plasmochin, and atebrin had very little or no inhibitory effect, even in fairly high concentrations. Prostigmin and phosphate esters[3] were the only substances having a high inhibitory coefficient in our experiments.

Exposure to 60° was found to be the most certain way to inhibit the enzyme activity (Fig. 2). It was used in our method because it gives the most complete inactivation; moreover, since the method is based on pH measurement, it is undesirable to use chemical agents which might cause changes of pH. We prefer the two-tube test to the one-tube method[5, 7] because it was found that most of the reaction takes place in the first half of the incubation period (Fig. 3), so that a variation of a few minutes

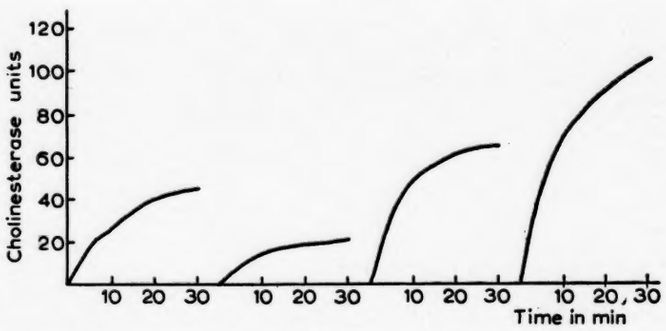

Fig. 3. Velocity of hydrolysis at 25°. (Most of the reaction takes place in the first half of the 30 min incubation period.)

in taking the "zero time" reading might cause considerable error. Moreover, even the finest colorimeter* might possibly give a different zero reading during a half hour period, thereby introducing another source of error. We have eliminated both error factors by the use of the method described.

The results obtained by the method are easily reproducible (Table II), and can be used for research investigations too. However, for more precise results the attachment of a Kipp galvanometer[9] to the colorimeter is advisable.

TABLE II

REPRODUCIBILITY OF THE METHOD

No. of specimen	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Ch. E. A. units on first examination	12	73	5	33	51	55	21	86	14	61
Ch. E. A. units on second examination	12	75	5	37	51	52	20	90	14	60
Ch. E. A. units of the same patient after 4–5 days	16	74	5	37	58	58	24	88	12	64

* We used the Klett-Summerson photoelectric colorimeter.

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It is very important to perform the test in an atmosphere free of the fumes of strong acids or ammonia. The absorption of CO_2 from the air is not a serious factor, since the change of pH it might cause is very slight in a strongly buffered substrate and, moreover, it will be the same both in the test and in its blank. Normal values found by our method were between 40–80 units. (Table III).

TABLE III

CHOLINESTERASE ACTIVITY OF SERUM OF NORMAL AND OF UNDERNOURISHED CHILDREN*

Group I Normal children Ch. E. A. units		*Group II Undernourished children 30–40% under weight Ch. E. A. units*			*Group II Undernourished children 40–50% under weight Ch. E. A. units*
37	66	58	26	45	12
32	34	9	65	37	40
45	35	6	41	20	32
40	48	16	18	40	5
40	36	52	55	40	20
24	63	48	20	35	12
56	78	13	14	34	15
30	52	50	28	20	14
38	86	44	14	5	10
62	70	22		34	
Average	50		31		18

* From Dr. MUNDEL's report (in preparation).

SUMMARY

An improved method for the measurement of serum cholinesterase activity is described. Its performance is easy and rapid, and gives reliable results.

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RECHERCHES SUR L'ACTION *IN VIVO* DU SULFATE DE PROTAMINE SUR LE SÉRUM HUMAIN

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Nous avons déjà décrit antérieurement[1-5] un aspect singulier de l'action du sulfate de protamine qui, administré par voie intraveineuse, provoque un abaissement du taux des lipides totaux et du cholestérol sanguin. Nous avons repris l'étude critique de cette action particulière: d'un part, en opérant une sélection très stricte des malades hyperlipidémiques, et en les soumettant à un protocole expérimental rigoureux; d'autre part, en nous assurant de la reproductibilité de nos dosages et les comparant avec les résultats obtenus à l'aide d'autres méthodes. Enfin, nous avons utilisé des protamines de provenances différentes.

PROTOCOLE EXPÉRIMENTAL

a) Choix des malades

Afin de limiter les possibilités de fluctuations biologiques spontanées, nous avons adopté d'emblée certaines précautions essentielles. Nous avons tout d'abord maintenu les sujets en état de jeûne absolu depuis au moins 10 heures, sans prolongation abusive, celle-ci étant par elle-même susceptible de favoriser une mobilisation des réserves lipidiques périphériques. Nous avons d'autre part, éliminé de notre étude les *hyperlipidémies "hyperlipémiques"* à sérum lactescent (comme l'hyperlipémie essentielle, les hyperlipémies aiguës qui peuvent se rencontrer au cours de l'acidose diabétique, dans la première phase d'une hépatite infectieuse ou virale, au cours des traitements cortisoniques, etc.), ainsi que les hyperlipidémies chroniques des diabétiques sans équilibration suffisamment rigoureuse du régime ou de l'insulinothérapie. Toutes ces formes cliniques d'hyperlipidémies présentent une dépendance trop manifeste vis-à-vis des variations du régime alimentaire pour se prêter à une étude expérimentale.

Nous avons choisi de préférence les hyperlipidémies à sérum clair, telles la xanthomatose hypercholestérolémique essentielle, l'hypercholestérolémie essentielle, affections connues comme étant beaucoup moins directement influencées par le régime alimentaire.

Enfin, dans les cas étudiés, toute interférence thérapeutique autre que le sulfate de protamine a été systématiquement éliminée, à l'exception toutefois de l'insulinothérapie dans certains cas d'hypercholestérolémie de diabétiques équilibrés de façon parfaite et de longue date.

b) Techniques employées

De nombreux travaux ont été consacrés aux problèmes des fluctuations biologiques spontanées de la cholestérolémie. Citons ceux de TURNER ET STEINER (technique de BLOOR)[6], de STEINER ET ROMANSKI[7], de MAN ET PETER[8] et enfin de SPERRY[9]

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(technique de SPERRY ET SCHOENHEIMER). Bien que ces études soient réalisées à l'aide de techniques de dosage différentes, les variations de la cholestérolémie étudiées pendant des mois n'excèdent guère, en général, pour un sujet donné, le taux de 15%. WATKIN, LAWRY, MANN ET HALPERIN[10] trouvent que la variation biologique du taux de cholestérol dans les hypercholérolémies intervient en fait faiblement et n'augmente pas, comme les variations d'origine technique, proportionnellement au taux du cholestérol. Ces auteurs montrent qu'avec des intervalles de trois jours séparant chaque dosage, un seul dosage présente 67% de probabilité d'avoir une déviation maxima de 15 cg p. 1000 de la valeur réelle; cette déviation est réduite à 10 cg p. 1000 pour deux dosages, à 5 cg p. 1000 pour 7 dosages.

Afin de réduire à leur minimum ces risques de fluctuations et d'en assurer en même temps le contrôle, nous nous sommes arrêtés au protocole expérimental suivant. Durant toute la période d'étude, qui comporte au minimum 7 à 8 jours, les lipides sériques du sujet sont quotidiennement contrôlés le matin à jeun à la même heure, après 10 heures de jeûne. 20 ml de sang sont prélevés sous garrot et recueillis sans anticoagulant; on laisse la coagulation s'effectuer spontanément à la température ambiante. Après rétraction du caillot, le sang est centrifugé 5 min à 3000 tours/min et le sérum décanté. Dans les minutes qui suivent le prélèvement, le patient reçoit une injection intraveineuse de 10 ml d'une solution limpide qui lui est constamment présentée comme contenant le produit actif, mais qui, pour les trois premiers contrôles au moins, en est dépourvue. On substitue ensuite à cette solution-témoin la solution de sulfate de protamine à étudier. Un tel protocole nous a paru présenter l'avantage de disposer ainsi: d'une période initiale de contrôle, d'une période d'exploration de l'action thérapeutique, et d'une période finale de contrôle post-thérapeutique.

Nous avons déterminé, chez les sujets étudiés, le taux des protéides et des lipides totaux (techniques pondérales), du cholestérol total (technique de GRIGAUT[11]), et la valeur du test phénolique de KUNKEL[12]. La plupart des patients étudiés étaient ambulatoires; de ce fait, ils n'étaient soumis à aucun contrôle strict de leur régime, astreints seulement à respecter leur régime habituel sans modifications intempestives de sa valeur calorique ou de sa teneur en graisses.

ÉTUDE CRITIQUE DE LA VALIDITÉ DES TECHNIQUES DE DOSAGES EMPLOYÉES

On sait à quelles difficultés et à quelles critiques théoriques un dosage aussi communément répandu que celui du cholestérol sanguin se heurte encore actuellement.

Bien que les méthodes hautement spécifiques du type SPERRY–SCHOENHEIMER, couplant précipitation à la digitonine et coloration de LIEBERMAN–BURCHARD, seraient *a priori* souhaitables, elles ne semblent pas finalement les meilleures en pratique sur le plan de la reproductibilité, en raison de la relative complexité des manipulations imposées. Ce fait ressort notamment des contrôles effectués sur des milliers de dosages, au cours de l'étude coopérative des lipoprotéines et du cholestérol pratiquée pendant 4 ans aux U.S.A. par 5 laboratoires conjugués[26]. La standardisation aboutit finalement aux choix de techniques plus simples du type de celle d'ABELL. La technique de GRIGAUT, qui garde tous ses droits et toute sa valeur en France, n'en est guère éloignée dans son principe. C'est pourquoi nous lui sommes restés fidèles dans cette étude. Les dosages présentés ici ont toujours été pratiqués dans le même laboratoire par la même laborantine, afin d'exclure le coefficient de variations personnelles.

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Afin de compléter ces garanties, 29 échantillons de sérum inclus dans cette étude ont été contrôlés dans un autre laboratoire au moyen d'une extraction différente des lipides (technique de DELSAL au méthylal–méthanol)*. La comparaison des résultats obtenus par les deux techniques pour les dosages du cholestérol total et des lipides totaux a été soumise à une étude statistique. Les résultats obtenus présentent un écart constant de 0.39 ± 0.01 g p. 1000 pour le cholestérol, les nombres obtenus par la technique de GRIGAUT étant les plus forts. Pour les lipides totaux, un écart constant de 0.92 ± 0.04 g p. 1000 est également trouvé entre les deux techniques, les nombres obtenus par extraction au méthylal–méthanol étant les plus élevés. A cet écart près, les résultats ne différent pas significativement (t cholestérol $= 1.18$ ($n = 29$); t lipides $= 1.14$ ($n = 29$)**). La linéarité des résultats des techniques employées par nous (Y) a été vérifiée par rapport aux techniques de contrôle (X):

$$\text{pour le cholestérol: } Y = 0.98\,X + 0.48$$
$$\text{pour les lipides: } Y = 0.96\,X - 0.42$$

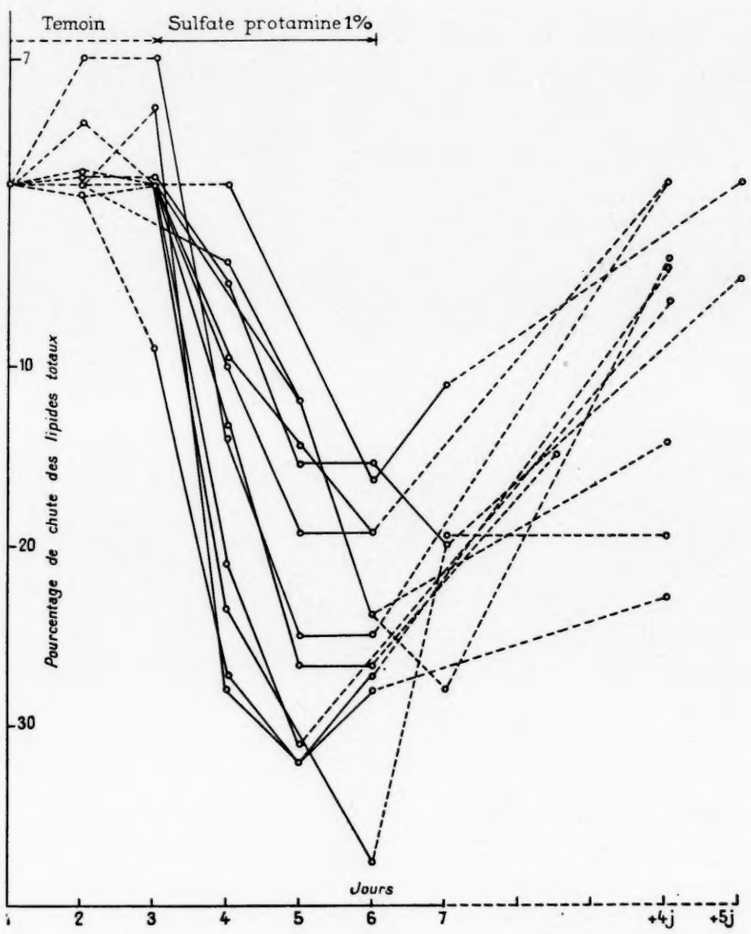

Fig. 1.

Fig. 1 – 2 – 3. Chaque point de la courbe en trait plein exprime la variation lipidique observée à la 24ème heure après injection intraveineuse de 100 mg de sulfate de protamine (10 ml à 1%).

* Ces contrôles ont été effectués dans le laboratoire du Prof. J. POLONOVSKI (Faculté de Médecine de Paris). Nous désirons le remercier ici de son aimable collaboration.

** t = test d'homogénéité.

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RÉSULTATS

Le Tableau I rassemble les résultats obtenus chez 11 malades atteints d'affections hyperlipidémiques diverses. Ils appellent quelques commentaires.

Nous notons une stabilité des concentrations en lipides totaux, du cholestérol total et du test de KUNKEL durant la première période de contrôle. Ces résultats confirment les prévisions issues des études statistiques de WATKIN, LAWRY, MANN ET HALPERIN[10] et apportent des données intéressantes concernant les variations spontanées de 24 h en 24 h de ces variétés d'hyperlipidémies. Au cours de la période d'exploration de l'action thérapeutique du sulfate de protamine[7]*, l'abaissement des taux des lipides totaux, du cholestérol total, du test de KUNKEL apparaît manifeste et parallèle. La progression de la baisse des lipides en fonction de la répétition des injections est fréquente (cas no. 3, 4 et 6), main non absolument constante. Les Figs. 1, 2 et 3, qui permettent de mieux étudier le phénomène, montrent que l'amplitude maxima de chute en 24 h se situe le plus souvent à la suite de la première injection.

Les contrôles pratiqués en période post-thérapeutique laissent déjà entrevoir une remontée précoce du taux lipidique, qui fut observée dans 3 des 5 contrôles pratiqués

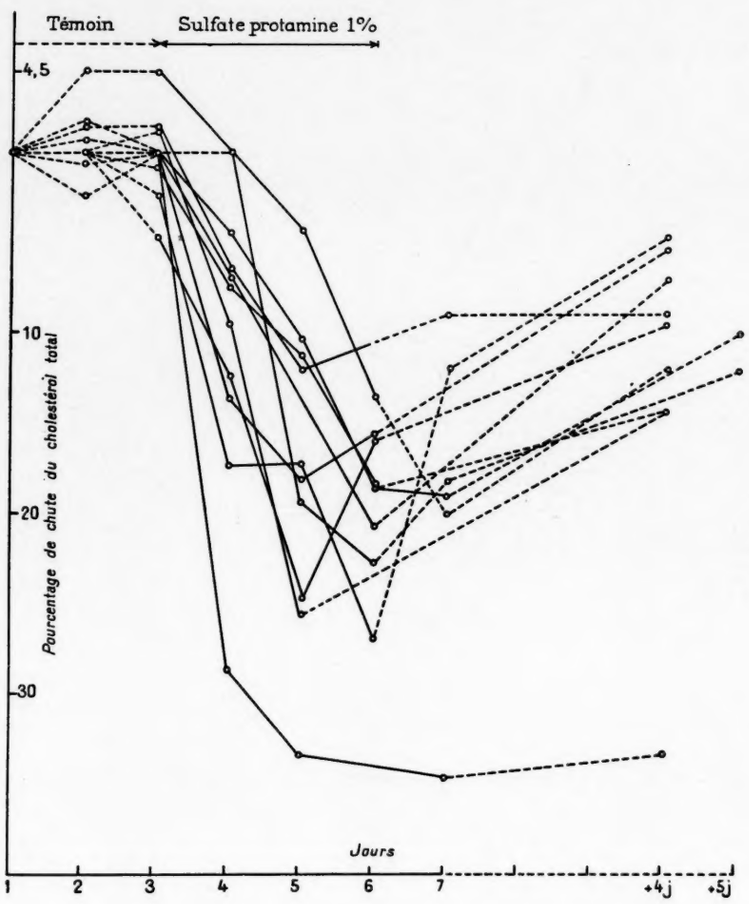

Fig. 2.

* Protamine Choay, lot no. 717 152.

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TABLEAU I

MODIFICATIONS QUOTIDIENNES DES TAUX DE CHOLESTÉROL ET DE LIPIDES CHEZ 11 MALADES SOUMIS À L'ACTION D'UN SULFATE DE PROTAMINE PAR VOIE INTRAVEINEUSE

No.	*Nom*	*Diagnostic*		*Témoin*			*Protamine*				*Contrôles post-thérapeutiques*	
1	Cor.	Diabète équilibré insuline	Protides g⁰/₀₀	80	80		80	80	80	80	+ 5 j.	80
			Lipides	9	9		9		7.5	8		9
			Chol. T.	3.3	3.3		3.3	2.66	2.55	2.7		2.9
			Kunkel	105	105		105	90	60	65		105
2	Per.	Hyperchol. essentielle	Protides g⁰/₀₀	80	82	80	80		79	79	+ 4 j.	80
			Lipides	10.5	10.6	10.5	8		6.5	8.5		8.5
			Chol. T	4.2	4.1	4.2	3		2.8	2.5		2.8
			Kunkel	70	70	70	60		50	60		70
3	Val.	Diabète équilibré insuline	Protides g⁰/₀₀	80	80	82	80	80	80	80	+ 4 j.	80
			Lipides	11	11	11.5	8	7.5	8	8.5		10.5
			Chol. T.	4.1	4.1	4	3.4	3.4	3	3.6		3.9
			Kunkel	115	115	115	100	96	76	80		110
4	Hal.	Xanthomatose tendineuse	Protides g⁰/₀₀	78.5	80	79	87	85	85	87	+ 4 j.	80
			Lipides	12.5	12.5		12	11	9.5	9		12
			Chol. T.	5.5	5.75	5.75	5.5	5.25	4.75	4.4		5
			Kunkel	190	185	190	160	105	105	100		190
5	Fou.	Hyperchol. origine X	Protides g⁰/₀₀	91	90	90	93	90	90		+ 4 j.	92.5
			Lipides	15.5	16	16	14	12.5	12.5			15.5
			Chol. T.	5.8	5.9	5.8	5	4.75	4.9			5.5
			Kunkel	167	163	160	120	90	70			92
6	Luc.	Syndrome néphrotique	Protides g⁰/₀₀	67	67	69	67	67		68	+ 4 j.	70
			Lipides	10.5	10	10.5	9.5	9		8.5		9
			Chol. T.	3.3	3.35	3.35	3.1	2.9		3		3
			Kunkel	75	72	73	65	50		45		60
7	Cus.	Athéromatose	Protides g⁰/₀₀	78	78	76	77.5	77			+ 4 j.	78
			Lipides	9.5	9.5	9.5	7.5	6.5				8
			Chol. T.	3.1	3.1	3.15	2.8	2.3				2.7
			Kunkel	70	70	72	50	40				45
8	Four.	Myxoedème	Protides g⁰/₀₀	84	85	84	84	84	84.5		+ 3 j.	84
			Lipides	14	15	15	12	10.5	10.5			14
			Chol. T.	5.8	5.75	5.8	5.4	5.2	4.6			5.4
			Kunkel	200	195	195	120	105	130			170
9	Pan.	Hyperchol. essentielle	Protides g⁰/₀₀	80	82	80	78	78	80	78	+ 4 j.	80
			Lipides	18	18	18	17	15	15	14.5		17
			Chol. T.	7.6	7.65	7.6	7.2	6.8	6.2	6.1		6.85
			Kunkel	346	350	346	242	180	165	150		270
10	Rou.	Hyperchol. essentielle	Protides g ⁰/₀₀	75	75	74	74	75	75		+ 4 j.	74
			Lipides	11	11	10	8	7.5	8			8.5
			Chol. T.	3.2	3.2	3.05	2.8	2.4	2.7			2.9
			Kunkel	66	66	66	55	52	60			67
11	Lef.	Xanthomatose	Protides g⁰/₀₀	72.5	72	70	72	72	70		+ 4 j.	70
			Lipides	15	15.5	15	13	11	11			14
			Chol. T.	5.4	5.4	5.35	5	4.8	4.4			4.85
			Kunkel	190	190	190	160	100	80			150

Témoin: 10 ml sérum physiologique isotonique.
Protamine: 10 ml sulfate de protamine Choay 1%.

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48 h après la dernière injection; lors du contrôle standard pratiqué le 4ème ou le 5ème jour après la dernière injection, la remontée est quasi-constante.

Étude comparative de l'action de sulfates de protamine d'origines différentes

Nous avons vérifié si la baisse du taux des lipides et du cholestérol était constante après injection de sulfate de protamine de provenances différentes. En effet, les modalités d'extraction et les critères de pureté du sulfate de protamine de provenances différentes. En effet, les modalités d'extraction et les critères de pureté du sulfate de protamine n'étant pas parfaitement définis, on peut se demander si l'action que nous avons décrite est générale.

Nous avons donc étudié sur une série de malades dont la sensibilité au sulfate de protamine avait déjà été éprouvée, l'action de différents sulfates de protamine (le produit leur étant présenté d'une manière identique quelle qu'en soit l'origine).

Les Tableaux II et III rendent compte des résultats obtenus.

Les sulfates de protamine de trois origines* montrent une action sensiblement équivalente. Au contraire, un sulfate de protamine d'origine américaine (Lilly) s'est révélé inactif dans les cinq cas étudiés.

La protamine inactive étant additionnée de 0.25 g de phénol pour 100 ml de solution, nous avons réalisé, pour préciser l'action inhibitrice éventuelle de ce produit, une série d'expériences dans lesquelles nous avons ajouté la même dose de phénol à un lot de sulfate de protamine reconnu actif par des expériences antérieures.

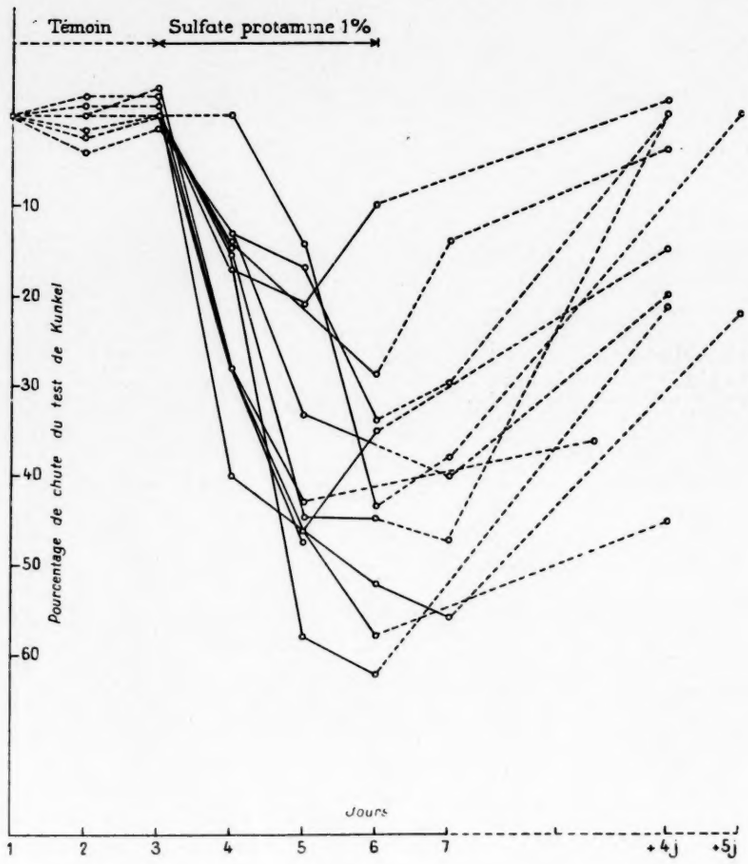

Fig. 3.

* Choay, Endopancrine, Novo.

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Sur les trois expériences ainsi pratiquées sur des patients hyperlipidémiques ayant antérieurement réagi positivement à un sulfate de protamine actif (Tableau IV), l'inactivation par le phénol est nette et confirmée de surcroît par la mise en évidence de l'activité du même produit sans phénol administré immédiatement à la suite de cet échec. Le mélange sulfate de protamine + phénol ayant été effectué extemporanément dans l'une des expériences, on ne peut apparemment mettre en cause une action dénaturante progressive, mais plutôt une inhibition immédiate de l'action physiologique.

TABLEAU II

RÉSULTATS OBTENUS AVEC DIFFÉRENTES PRÉPARATIONS DE SULFATE DE PROTAMINE

Nom	*Diagnostic*		*Témoin*			*Protamine R*			*Contrôles post-thérapeutiques*	
LEF. 3ème Exp.	Xanthomatose	Protides g⁰/₀₀	78	78	76	76	76	76	+ 4 j.	76
		Lipides	13	12.5	12.5	10.5	10	10		13
		Chol. T.	4.8	4.8	4.8	4.5	4.1	3.8		4.5
		KUNKEL	140	138	140	112	105	90		140
DOU. 2ème Exp.	Hyperchol. essentielle	Protides g⁰/₀₀	82	80	82	80	84	80	+ 4 j.	82
		Lipides	18	18	18	16	14	13		15
		Chol. T.	5.5	5.55	5.7	5.1	4.5	4.1		5
		KUNKEL	185	185	180	138	130	105		160

Témoin: 10 ml sérum physiologique isotonique.
Protamine R: 10 ml sulfate de protamine Roche à 1%.

Nom	*Diagnostic*		*Témoin*			*Protamine N*			*Contrôles post-thérapeutiques*	
PAN. 3ème exp.	Hyperchol. essentielle	Protides g⁰/₀₀	78	76	80	78	78	76	+ 5 j.	78
		Chol. T.	5.8	5.8	5.8	5	4.8	4.1		5
		KUNKEL	150	145	155	120	100	90		110
LEF. 4ème exp.	Xanthomatose	Protides g⁰/₀₀	72	70	74	76	72	72	+ 4 j.	74
		Chol. T.	4.8	4.85	4.75	4.1	3.8	3.5		4
		KUNKEL	150	152	152	110	90	83		85

Témoin: 10 ml sérum physiologique isotonique.
Protamine N: 10 ml sulfate de protamine Novo à 1%.

Nom	*Diagnostic*			*Témoin*			*Protamine L*	
FOU. 2ème exp.	Hyperchol.	Lipides g⁰/₀₀	Extraction méthylal–méthanol	17.5	17.3	15.9	15.5	15
			Ext. ether	16	16	16.5	16	16
			Ecart	—1.5	—1.3	+0.6	+0.5	+1
	Origine ?	—d⁰—	Extraction méthylal–méthanol	5.4	5.35	5.5	4.6	4.1
			Ext. ether	5.6	5.6	5.5	5.6	5.6
			Ecart	+0.20	+0.25	0	+1	+1.5

Témoin: 10 ml sérum physiologique isotonique.
Protamine L: 10 ml sulfate de protamine Lilly à 1%.

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TABLEAU III

ÉTUDES COMPARATIVES SUCCESSIVES CHEZ UN MÊME SUJET DE DEUX PRÉPARATIONS DE SULFATE DE PROTAMINE

Nom	*Diag.*		*Témoin*			*Protamine L+*			*Protamine CH++*			*Contrôle post-thérapeuti*	
Per.	Hyper-	Protides g⁰/₀₀	82	80	80	80	80						
2ème	chol.	Lipides	7	7	7	7	7						
exp.	essent.	Chol. T	2.8	2.8	2.8	2.75	2.75						
		Kunkel	70	68	70	73	73						
Fou.	Hyper-	Protides g⁰/₀₀	90	91	92	91	92		94	92		+ 4 j.	92
2ème	chol.	Lipides	16	16	16.5	16	16		15.5	16			16
exp.	origine?	Chol. T	5.6	5.6	5.55	5.6	5.6		5.3	5			5
		Kunkel	120	120	115	120	120		100	95			125
Lef.	Xantho-	Protides g⁰/₀₀	80	80	82	78	78	78	82	78	78	+ 4 j.	80
2ème	matose	Lipides	15	16	16	16	16.5	16	14	12	12		13
exp.		Chol. T	6	6	6	6.1	6	6.1	5.4	5.2	5.1		5
		Kunkel	178	173	170	175	180	196	130	110	100		170
Pan.	Hyper-	Protides g⁰/₀₀	78	77	78	78	78	78	78	78	78	+ 3 j.	77
2ème	chol.	Lipides	17	18	18	18	17.5	18	17	15.5	15		15
exp.	essent.	Chol. T	7	7.1	7	7.1	6.9	7	6.5	6.3	5.8		6
		Kunkel	196	210	210	208	210	200	160	156	140		170
Dou.	Hyper-	Protides g⁰/₀₀	84	84	84	84	82	82	85	85	85	+ 5 j.	87
1ère	chol.	Lipides	15.5	15	15	15.5	15	15.5	13	12	13.5		13
exp.	essent.	Chol. T	5.8	5.75	5.5	5.5	5.5	5.8	5.1	4.7	4.6		5
		Kunkel	135	135	140	135	135	140	110	105	95		110

Témoin: 10 ml sérum physiologique isotonique.
Protamine L+: 10 ml sulfate de protamine Lilly à 1%.
Protamine CH++: 10 ml sulfate de protamine Choay à 1%.

TABLEAU IV

Nom	*Diag.*		*Témoin*			*Protamine CH + P*			*Protamine CH*			*Contrôle post-thérapeutique*	
Dou.	Hyper-	Protides g⁰/₀₀	80	82	80	82	82	82	82	80	80	+ 4 j.	82
3ème	chol.	Lipides	15	15.5	15	15	16	15	14	13	13		14
exp.	essent.	Chol. T.	5.3	5.25	5.25	5.1	5	4.75	4.1	3.9	3.7		4.2
		Kunkel	160	155	155	152	150	160	132	120	110		140
Lef.	Xantho-	Protides g⁰/₀₀	78	77	78	78.5	76	78.5	78	78.5	78	+ 4 j.	79
5ème	matose	Lipides	13.5	13	13.5	13.5	13.5	14	13	12	11		13
exp.		Chol. T.	4.5	4.4	4.5	4.4	4.4	4.4	4	3.7	3.35		3.75
		Kunkel	146	145	148	150	148	158	120	110	95		115
All.	Diab.	Protides g⁰/₀₀	75	75	74	74	76	74	74	76		+ 3 j.	74
1ère	hyper-	Lipides											
exp.	chol.	Chol. T.	3.1	3.15	3.2	3.2	3.2	3.2	2.75	2.5			2.6
		Kunkel	105	110	105	100	105	90	70	50			57

Témoin: 10 ml sérum physiologique isotonique.
Protamine CH + P: 10 ml sulfate de protamine à 1% Choay + 25 mg de phénol.
Protamine CH: 10 ml sulfate de protamine à 1% Choay.

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DISCUSSION

De nombreux travaux ont déjà été consacrés à l'étude de l'action du sulfate de protamine sur les lipides sanguins. L'effet le plus souvent reconnu est une action hyperlipémiante. Le rôle inhibiteur du sulfate de protamine vis-à-vis du phénomène de clarification par l'héparine est désormais classique (BROWN[19]; SWANK ET ROTH[20]; SPITZER[21]; BROWN, BOYLE ET ANFINSEN[22]. Cette inhibition, qui s'exerce tant *in vivo* qu'*in vitro*, est capable, suivant le moment de son intervention, de prévenir ou de freiner l'évolution normale de la clarification. Bien plus, un effet réversible secondaire, capable de restituer au sérum sa turbidité originale avant clarification, peut s'observer. La raison de cette inhibition est diversement interprétée et a pu être tour à tour imputée: a) une neutralisation directe de l'héparine en tant que coenzyme par la charge électrique fortement basique de la protamine (ANFINSEN[16]); b) à une inhibition directement enzymatique de la lipoprotéine-lipase (KORN[14]); c) à une neutralisation indirecte *in vivo* par l'intermédiaire des surrénales et de l'hypophyse (SEIFTER)[17]; d) ou encore peut-être à une combinaison directe catio-anionique avec les acides gras libres (LAUDAT ET DE GENNES)[5].

Lorsque son action est envisagée isolément, le sulfate de protamine est aussi généralement considéré, – tout au moins *in vivo*, – comme un agent provoquant l'augmentation des lipides et des lipoprotéines circulantes (BROWN[22]; SZASZ ET CONSTANTINIDES[23]; BRAGDON ET HAVEL[24]).

En ce qui concerne la régression spontanée de la lipémie postprandiale, SPITZER a montré son ralentissement net par l'injection de sulfate de protamine, voire la réapparition secondaire de la lipémie dans les cas où celle-ci s'était déjà pratiquement effacée (SPITZER[25]; SWANK ET ROTH[20]).

A l'exception de nos travaux personnels, seuls ceux de GROSSMAN et coll.[15] signalent une action hypolipémiante et décrivent une accélération de la fuite extravasculaire des lipides après introduction intraveineuse simultanée d'émulsion graisseuse et de sulfate de protamine. Les auteurs attribuent ce fait à un effet d'agglutination du sulfate de protamine sur les chylomicrons, et à une épuration sanguine accélérée de ces graisses devenues grossièrement particulaires par les cellules macrophages du système réticulo-endothélial, notamment les cellules de KÜPFFER du foie et de la rate. Mais encore cette éventualité apparaît-elle bien restreinte, réduite aux strictes conditions du protocole expérimental utilisé, et ne jouant apparemment dans ce cas que sur le transport des chylomicrons. Aucune allusion n'est faite par GROSSMAN et coll. à une action analogue sur le cholestérol et les lipides transportés dans les β-lipoprotéines.

L'indication de cette dernière éventualité restant donc une position très personnelle, les contrôles présentés dans ce mémoire relèvent du souci de vérifier très soigneusement nos assertions.

D'après nos résultats, nous ne pensons pas qu'un simple artefact dans l'observation des phénomènes, qu'il soit d'ordre technique ou biologique, puisse représenter une explication suffisante de cette apparente divergence d'opinion. Dans sa genèse, d'autres facteurs sont susceptibles d'être intervenus, parmi lesquels l'origine des sulfates de protamine employés, ou bien encore des différences dans les repères chronologiques adoptés.

En ce qui concerne la première éventualité, nous pensons pouvoir exclure l'hypo-

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thèse de l'interférence *accidentelle* d'une impureté active contaminant les préparations de sulfate de protamine actives. En effet, l'action s'observe avec toute une série de préparations d'origines différentes, et s'il s'agissait d'une impureté active, on devrait admettre que son association avec le sulfate de protamine ne relève pas d'un simple hasard. Il semble plus logique d'attribuer, au contraire, aux substances étrangères l'inactivité de certaines préparations: nos résultats démontrent en effet l'action inhibitrice du phénol, présent dans la seule solution de sulfate de protamine qui nous ait donné des résultats négatifs.

Restent encore, comme explication possible des discordances, les différences chronologiques dans l'observation des faits. Dans la plupart des travaux, l'action du sulfate de protamine *in vivo* est étudiée dans un délai assez bref, dépassant rarement deux heures. Dans nos expériences, au contraire, ce délai est très sensiblement prolongé et les variations caractéristiques n'apparaissent qu'à partir de la 3ème ou de la 4ème heure après l'injection (DE GENNES[18]), son amplitude maxima se situant à la 24ème heure.

Nous ignorons naturellement le mécanisme de l'action particulière du sulfate de protamine, mais les faits que nous rapportons nous semblent suffisamment nets pour retenir l'attention et susciter de nouvelles recherches.

RÉSUMÉ

Pour préciser l'action du sulfate de protamine sur les lipides circulants, décrite dans nos travaux antérieurs, une révision critique de cette action a été entreprise sur une nouvelle série de 11 sujets hypercholérolestémiques soigneusement sélectionnés, présentant des hypercholestérolémies permanentes à sérum clair, peu sensibles aux variations du régime alimentaire et habituellement assez stables. Un protocole expérimental rigoureux, comportant des contrôles quotidiens de la lipidémie et la mise en œuvre de techniques différentes, a été adopté. Il a ainsi été possible:

(1) de faire la part des fluctuations quotidiennes *spontanées* de la cholestérolémie;

(2) de confirmer les diminutions parallèles de la lipidémie totale, de la cholestérolémie et du test phénolique de KUNKEL, consécutives aux injections intraveineuses de sulfate de protamine, la diminution dépassant 15% après 3 jours pour la cholestérolémie;

(3) d'établir une relation entre l'inactivité de l'une des préparations de sulfate de protamine étudiées et la présence de phénol.

Les auteurs attribuent aussi la discordance apparente entre les effets observés et les effets classiquement admis du sulfate de protamine au délai plus long écoulé dans leurs observations entre l'injection et le prélèvement sanguin.

SUMMARY

ACTION OF PROTAMINE SULPHATE ON HUMAN SERUM *in vitro*

In order to obtain more precise information about the action of protamine sulphate on serum lipids, described previously by the authors, another series of eleven carefully selected cases of hypercholesterolaemia was observed. All the patients had permanent cholesterolaemia with clear sera, which was not very sensitive to changes in diet and in general remained fairly stable. An experimental schedule was strictly

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followed; it included daily checks of the lipaemia as well as various other determinations. In this manner it was possible:

(1) to follow the daily *spontaneous* fluctuations of the cholesterolaemia;

(2) to verify the parallel diminution of the total lipaemia, the cholesterolaemia and the phenol test of Kunkel following the i.v. injection of protamine sulphate, whereby the cholesterolaemia was found to decrease by more than 15% after 3 days;

(3) to establish a relationship between the inactivity of one of the protamine sulphate preparations studied and the presence of phenol.

The authors attribute the apparent disagreement between the effect of protamine sulphate observed by them and those described in the literature to the longer time that elapsed between injection and withdrawal of blood in their experiments.

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RAPID TURBIDIMETRIC METHODS FOR THE DETERMINATION OF PLASMA FIBRINOGEN

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Acute haemorrhage due to hypofibrinogenaemia is an emergency which calls for a simple rapid method for the determination of plasma fibrinogen. Such a method has been in use in this department for some time and is presented here.

The turbidity produced by addition of a sodium sulphate solution to plasma is measured using the M.R.C. grey wedge photometer. Other turbidimetric methods for fibrinogen have been published: PARFENTJEV, JOHNSON AND CLIFFTON[1] described a method using ammonium sulphate as salting out agent. STIRLAND[2] precipitated fibrinogen by incubation at 56° in sodium chloride solution. YEOMAN[3] has described the use of the grey wedge photometer for turbidity measurement. These four methods devised have been compared with the classical CULLEN AND VAN SLYKE[4] Kjeldahl procedure.

METHODS

Oxalated plasma (2 mg potassium oxalate per ml blood) was used throughout and was obtained from specimens coming into the laboratory for a variety of routine procedures.

Plasma fibrinogen was determined by the following methods:

1. Method as used in this laboratory

To 0.5 ml plasma was added 5.0 ml sodium sulphate solution (105 g anhydrous Na_2SO_4/l solution). A control, 0.5 ml plasma to 5.0 ml sodium chloride (0.9 g/100 ml) was also put up. Each tube was mixed, left to stand 3 min, mixed by gentle inversion and read in the grey wedge photometer using the green filter (Ilford 625) at 1 cm optical depth. The difference in reading between test and control is a measure of the fibrinogen present. All readings on this instrument were made with the compensating cell omitted from the left hand side of the instrument. This procedure ensured that readings did not fall below 15 degrees, the value below which readings are liable to be inaccurate.

2. Procedure of Parfentjev et al.

To 0.25 ml plasma was added 0.25 ml sodium chloride solution (0.9 g/100 ml) and 4.5 ml ammonium sulphate solution (13.33 g/100 ml), optical density was measured in the Unicam S.P. 600 spectrophotometer, 1 cm optical depth, 450 mμ. A control, 0.25 ml plasma to 4.75 ml 0.9% sodium chloride solution, was also read.

3. Procedure of Parfentjev et al. modified

In this laboratory, the method was modified for measurement in the grey wedge photometer by adding to 0.5 ml plasma, 4.5 ml 13.33% ammonium sulphate. Ilford

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filter 625, 1 cm optical depth used. A control, 4.5 ml 0.9% sodium chloride to 0.5 ml plasma was also read.

4. Procedure of Stirland

A 1/16 dilution of plasma in sodium chloride (1.0 g/100 ml) was used. An aliquot was incubated at 56° for 15 min, the remainder of the dilution serving as the control. Measurement was made in the Unicam SP. 600 spectrophotometer, 1 cm optical depth at 650 mμ.

5. Reference method

Recalcification of plasma and isolation of fibrin clot according to CULLEN AND VAN SLYKE[4] followed by Kjeldahl digestion and distillation.

CALCULATION OF RESULTS

For each turbidity method the fibrinogen content is related to the difference in optical density between the test and the control. The plasma fibrinogen is found from this difference by reference to a calibration curve constructed for each method using serial dilutions of plasma of fibrinogen content determined by a standard method. The dilutions are made in serum. Instead of a calibration curve an artificial turbidity standard such as barium sulphate (KUNKEL[5]) or formazin (KINGSBURY *et al.*[6]) may be used after standardization against the turbidity produced by known amounts of fibrinogen.

The preparation of the turbidity standard must however be carefully standardized; for instance in the preparation of the barium sulphate standard, the optical density of the suspension obtained by the addition of the sulphuric acid to the barium chloride may differ by as much as 25% from that obtained by addition of the barium chloride to the acid. The technique used in mixing the two reagents must be standardized and constantly adhered to in order to obtain reproducible standard readings.

RESULTS

Fibrinogen determinations were carried out by all five methods in duplicate on samples of plasma from 24 patients. The results are shown in Fig. 1 and a statistical summary is shown. The fibrinogen content of the plasma ranged from 0.12 to 0.50 g/100 ml (mean 0.327 g/100 ml).

DISCUSSION

The results show that the correlations of the turbidity methods with the Kjeldahl procedure are similar. In assessing the results it is assumed that the fibrinogen value yielded by the Kjeldahl procedure is the true plasma fibrinogen, with so many factors contributing to clot formation and sometimes clot lysis, this assumption may be more inaccurate than is often supposed.

It will be noted that for all turbidity methods the regression curves (Fig. 1) do not pass through the origin. To substantiate this observation, pooled plasma (fibrinogen content determined by Kjeldahl) was diluted serially with serum to which potassium oxalate had been added and the precipitated calcium oxalate removed by centrifugation. Each turbidimetric method was carried out on each dilution and

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optical density plotted against fibrinogen content. A straight line drawn through the points in no case passed through the origin. For each method turbidity occurs only when a given plasma fibrinogen value is exceeded: these values were approximately: Method (1) 0.06 (2) 0.09 (3) 0.07 (4) 0.15 g/100 ml. If the fibrinogen content is low, STIRLAND uses a higher concentration of plasma in saline for the determination.

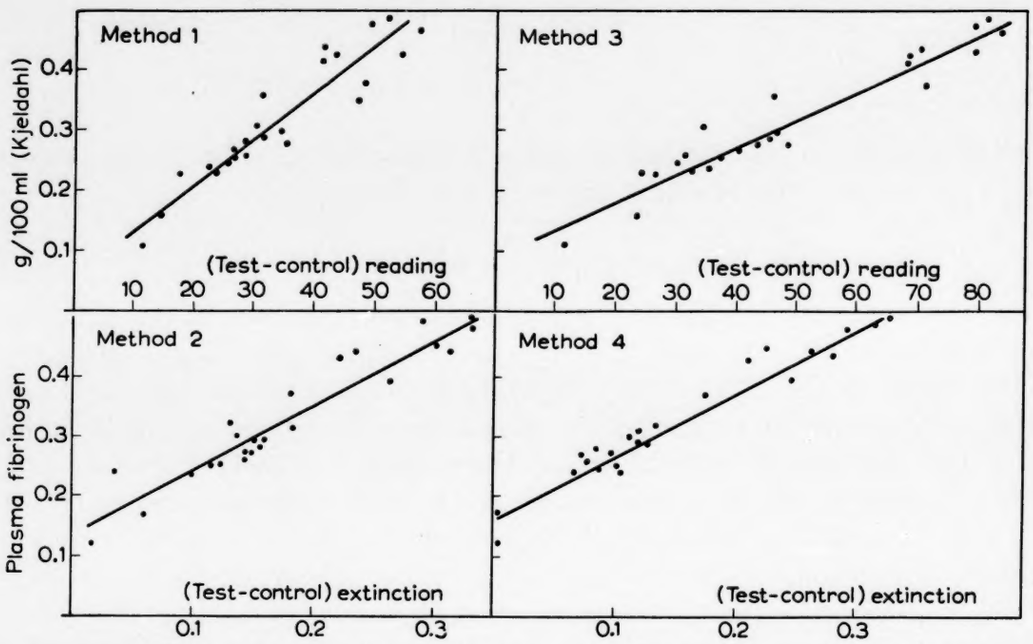

Fig. 1. Comparison of each turbidimetric method with Kjeldahl fibrinogen values (24 plasma). Methods: (1) Present method; (2) PARFENTJEV *et al.*; (3) PARFENTJEV modified for grey wedge photometer; (4) STIRLAND.

Among the turbidity methods small advantages emerge. The three salting out procedures require about 5 min, STIRLAND's method takes some 25 min. The STIRLAND procedure and the modified method of PARFENTJEV *et al.* correlate a little better with the Kjeldahl values. The methods using the grey wedge photometer are well suited for use in blood transfusion and haematology laboratories, the department most

TABLE I

COMPARISON OF THE FIVE METHODS FOR THE DETERMINATION OF PLASMA FIBRINOGEN

	Method				
	1	*2*	*3*	*4*	*5*
Standard deviation by analysis of variance of duplicates for each method ± g Fibrinogen/100 ml	0.011	0.019	0.0123	0.021	0.011
Correlation coefficient between each method and Kjeldahl fibrinogen	0.945	0.952	0.964	0.972	
Standard error of each method assuming Kjeldahl value to be the true fibrinogen content ± g Fibrinogen/100 ml plasma	0.033	0.033	0.028	0.025	

Methods: (1) Present method; (2) PARFENTJEV *et al.*; (3) modified PARFENTJEV; (4) STIRLAND; wedge (5) Kjeldahl..

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concerned at the time of an emergency acute haemorrhage. The Zeiss- Pulfrich photommeter would also seem quite suitable for the turbidity measurements.

In addition to their rapidity, the turbidity methods are very useful for investigations of plasma containing fibrinolysins where results obtained by methods involving analysis of a fibrin clot may be invalid.

ACKNOWLEDGEMENTS

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SUMMARY

Four rapid turbidimetric methods for plasma fibrinogen determination are compared with the standard Kjeldahl procedure. It is concluded that the turbidimetric methods are of sufficient accuracy and reliability as to be suitable for emergency and routine work.

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ALBUMIN TRAIL IN PAPER PROTEIN ELECTROPHORESIS

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The advent of electrophoresis has provided an elegant method for testing and comparing the efficiency of salt fractionation techniques. Such comparative work[1-4] has shown that none of the established methods is capable of producing completely accurate separation over the wide range of normal and abnormal concentrations of protein met in everyday clinical work.

The introduction of paper protein electrophoresis has made possible a fractionation technique which is independant of the hazards of the chemical procedures of separation.

Since the practice of paper electrophoresis is relatively simple, there would now seem to be a very good case for the abandonment of all salt fractionation techniques in favour of this newer method.

Several workers have observed that the filtration methods of separation gave rise to errors in salt fractionation technique, and concluded that adsorption occurred at the filter paper interface[5-7]. The possibility that this might occur in paper electrophoresis was noted by MERKLEN AND MASSEYEFF[8] in 1952.

JENCKS and his associates[9] have readily appreciated that such trailing does occur, and have suggested that it approximates to between 3 and 5% of the total albumin. They have indicated that a correction of this order might be applied.

Reference to the original paper of the previous workers shows that in the two series of albumin fractions which they examined, the degree of albumin trailing amounted to about 24% using Whatman No. 1 paper, about 27% on Whatman No. 2, and about 49% on Munktell No. 20. It may readily be appreciated that considerable differences in trailing might occur on papers which have physical characteristics as divergent as Whatman No. 1 and Munktell No. 20.

The object of the present series of experiments was to try to decide whether the degree of albumin trailing was

(a) a constant percentage of the total albumin, or

(b) represents a constant quantity of albumin.

As a result of the findings a method was derived to assess the amount of trailing.

MATERIAL AND METHODS

Buffers

Throughout the experiments the barbitone–barbituric acid buffer of DURRUM[10] was used; this was of ionic strength (I) 0.05, and pH 8.6. Other buffers were investigated including: barbitone–barbituric acid pH 8.6, $I = 0.10$; veronyl–acetate buffer pH 8.6, $I = 0.084$; sodium–potassium phosphate buffer pH 7.4, $I = 0.10$; sodium acetate–acetic acid buffer pH 5.2, $I = 0.075$; sodium acetate–acetic acid pH 5.2, $I = 0.040$; equivolume mixture of sodium acetate–acetic acid buffer pH 5.2 and

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barbitone buffer pH 8.6, final pH of mixture 8.0, I not determined. Barbitone buffer pH 8.6, $I = 0.05$, to which 0.384 g/l of calcium lactate had been added[11]; the borate buffer of CONSDEN AND POWELL[12].

Dye substances

Naphthalene Black 12B (Imperial Chemical Industries Ltd), Azocarmine B, Bromophenol Blue, Lissamine Green G, Azocarmine GX, Bromophenol Green and Light Green (all these from Messrs. Geo. T. Gurr, London).

Albumin

The albumin used in the recovery experiments was supplied by the Blood Products Laboratory of the Lister Institute, Elstree, Herts. This fraction is prepared according to the method of KEKWICK AND MACKAY[13] using an ether gradient, and is of at least 97% purity. For the purpose of this experiment it was further subjected to electrophoresis and concentration, the initial albumin of 'trail'—which might contain up to 3% of contaminating globulins—was therefore avoided.

Electrophoresis

This was performed in horizontal chambers of minimal air space above the strip, temperature variation was minimal although the chambers were not water cooled. The voltages applied were varied from 80 V, by 30-V intervals to 170 V; over this range the current remained almost constant at 0.75 mA per strip. In the higher ranges of voltage, observations were made at 60-V intervals from 210 V to 450 V, the current density varying within this range from 1.0 mA to 1.8 mA per strip.

The filter paper strips were generally Whatman No. 1 chromatography paper, although a few experiments were also conducted using Whatman 3 MM, and Whatman "Milk-strip" extra thick papers. Separations were also effected using cellulose acetate membranes[14], the product of Oxo Ltd. All strips were cut to 34 × 5 cm in dimension before use. Whatever type of paper was used, equilibrating periods of 30 min were allowed in the electrophoretic cell before application of serum. The electropherograms were "run" for a period of 16 h excepting in the higher ranges of voltage, or when "short" separations of from 2½–6 h were effected.

Scanning of stained preparations was made by transmittance methods using an Eel scanner, or by reflectance methods using a Joyce Loebl photodensitometer. Areas beneath scanned curves were computed either by means of a planimeter, or by mathematical reduction to trapezia.

As far as possible, sera were used within 4 h of taking the sample. No serum was used which had been previously refrigerated.

RESULTS

Fig. 1 represents a series of tracings of increasing concentrations of pure albumin solution. In ascending order the absolute quantities applied to the paper were: 0.05 mg, 0.11 mg, 0.23 mg, and 0.46 mg. If a normal serum is considered at a protein concentration of 7.0 g%, then seeding of 0.010 ml of serum for the purpose of experiment infers that the absolute quantity of protein being subjected to electrophoresis is 0.70 mg. Expressed as percentages of 0.70 mg the above quantities of albumin represent an increasing proportion of 7.14%, 15.71%, 32.86% and 67.14%

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respectively. The degree of albumin trail, expressed as a percentage of total albumin for this series was 49%, 28.6%, 21% and 18%; these values are equivalent to an increasing gradient of 0.025 mg, 0.031 mg, 0.048 mg, and 0.084 mg in absolute quantities respectively.

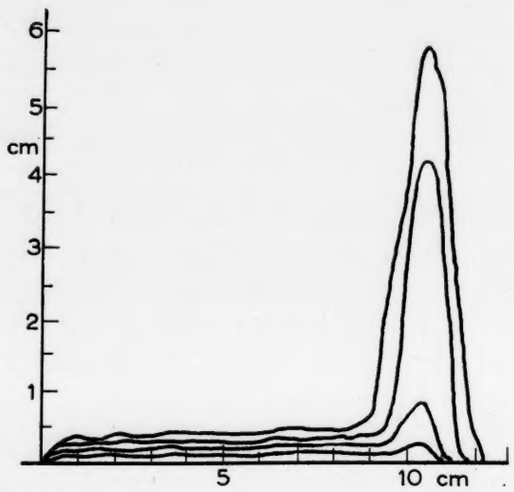

Fig. 1. Trailing in increasing concentration of pure albumin solution.

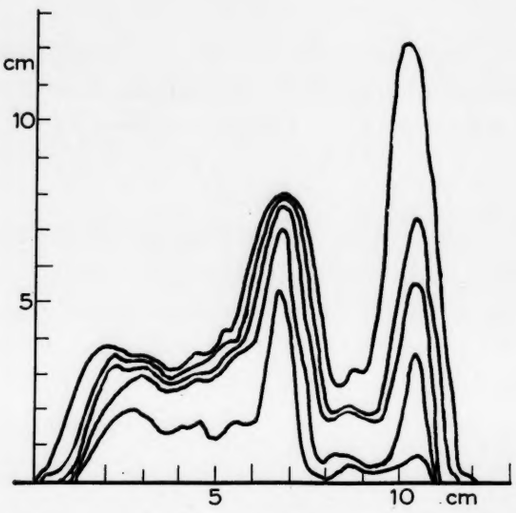

Fig. 2. Tracings obtained by addition of albumin to a nephrotic serum. The lowest curve represents the original to which 0.05 mg, 0.11 mg, 0.23 mg, and 0.46 mg of albumin have been successively added.

Fig. 2 represents tracings obtained by adding the same quantities of albumin to a sample of markedly hypoalbuminaemic serum from a patient with nephrosis. The albumin trail is now represented by the following series of percentages: Original serum 49%, after addition of 0.05 mg of albumin, 33%, after addition of 0.11 mg 29.2%, of 0.23 mg 19.9%, of 0.46 mg 20.1%.

The absolute recoveries of albumin in three similar series of experiments are shown in Table I. In these experiments 0.010 ml of the serum and of the albumin solution was applied to the paper which was cut in half before scanning in order to

TABLE I

RECOVERY OF ADDED ALBUMIN

Series	*Original albumin content (mg)*	*Amount of albumin added (mg)*	*Albumin expected (mg)*	*Albumin found (mg)*	*% Recovery*	*% of Albumin trail*
1 (a)	0.0300	0.0500	0.0800	0.0788	98.5	49
(b)		0.1100	0.1400	0.1504	107.4	31
(c)		0.2300	0.2600	0.2788	107.2	28
(d)		0.4600	0.4900	0.4864	99.3	26
2 (a)	0.0547	0.0500	0.1047	0.0958	91.5	46
(b)		0.1100	0.1647	0.1598	97.0	33
(c)		0.2300	0.2847	0.2758	96.9	20
(d)		0.4600	0.5147	0.5082	98.7	25
3 (a)	0.0807	0.0500	0.1307	0.1264	96.7	47
(b)		0.1100	0.1907	0.1909	100.1	29
(c)		0.2300	0.3107	0.3053	98.3	20
(d)		0.4600	0.5407	0.5327	98.5	20

Mean recovered in 3 series = 99.2%.

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maintain the optical density of the bands within the required limits for Beer's Law to be obeyed.

Fig. 3 shows a tracing of a normal serum, and compares the prestained (bromophenol blue) albumin trail with the fully stained protein spectrum.

Table II represents an investigation of two normal sera (N. 1 and N. 2) and two pathological sera (P. 1, another case of nephrotic syndrome, and P. 2, a case of staphylococcal pyaemia). Three dye substances were used, Azocarmine B[15], Naphthalene Black 12B[16], and Bromophenol Blue[17]. After scanning, the percentage of albumin was calculated in each case having (a) regard for albumin trail (A.T.A.), and (b) taking no account of albumin trail (No A.T.A.). The *A/G* ratios were computed for comparative purposes. It will be seen that there is a considerable difference between the values of albumin per cent when albumin trail is not allowed, and this difference contributes seriously to the computed *A/G* ratio.

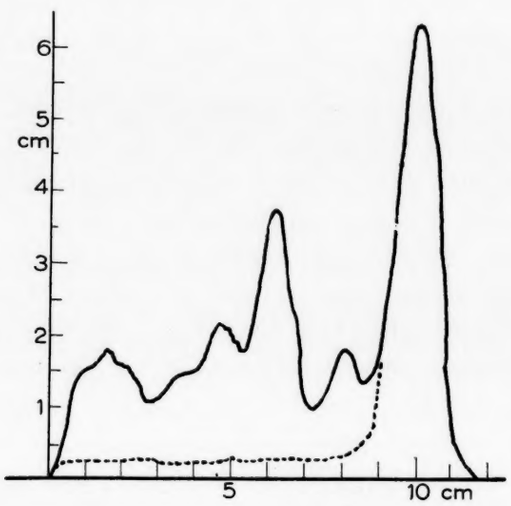

Fig. 3. Tracing of protein curve, prestained to show albumin trail, then fully stained to show protein dispersion (Bromophenol blue stain). The ratio of the ordinal heights at the α_2/α_1 globulin trough is 0.33.

TABLE II

Dye used	*Serum*	*Albumin %*		*A/G ratio (computed)*	
		A.T.A.	*No A.T.A.*	*A.T.A.*	*No A.T.A.*
Azocarmine B	N.1	55.2	48.6	1.23	0.95
	N.2	58.1	49.8	1.38	0.99
	P.1	26.2	19.1	0.36	0.24
	P.2	47.1	38.2	0.89	0.62
Naphthalene Black 12B	N.1	53.9	42.8	1.16	0.75
	N.2	57.6	48.8	1.36	0.95
	P.1	25.7	18.3	0.35	0.22
	P.2	46.8	39.2	0.88	0.63
Bromophenol Blue	N.1	53.3	44.8	1.14	0.81
	N.2	56.7	47.3	1.31	0.89
	P.1	27.3	22.2	0.38	0.29
	P.2	47.7	37.6	0.91	0.60

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DISCUSSION

From the results obtained it would appear that the degree of albumin trailing is never constant for any one serum or series of sera. In an investigation of two hundred normal sera the degree of albumin trail using Whatman No. 1 paper was found to lie generally between 9 and 14% of the total albumin; measured in absolute quantities this was never a constant amount.

A series of investigations such as that illustrated in Fig. 3 reveals that the depth of the albumin "carpet" measured from the abscissa of scan is almost exactly one third of the minimal height of the lowest trough obtained in the scan. In a series of over three hundred such investigations, the ratio indicated was 0.32 ± 0.2. This trough usually occurs between the α-globulin fractions, but may occur in other places.

If Whatman No. 3 MM paper is used the general order of trail is rarely less than 20% of the total albumin, and with thicker papers it may be even greater. The above mentioned ratio, however, seemed to remain constant even with these thicker papers.

If the albumin concentration is considerably diminished, as for example in the protein-losing pathological states, the degree of albumin trail may approach 50% of the total albumin.

During the course of experiments wherein rapid separation was effected using voltages of 400 V upwards (Whatman No. 1 paper), the degree of albumin trail increased, and was generally of the order of 24% of the total albumin in normal sera. Increase in ionic strength of buffer led to similar increases.

Neglect of allowance for albumin trail may lead to artificial depressions of albumin by as much as 10% of the total protein spectrum. This is more obvious in those cases where there is a true deficiency of albumin. Such a manifestation of artificial depression is obtained when automatic integrating instruments are employed which make no allowance for albumin trailing. Depression in albumin caused in this way is reflected in "falsely high" globulin percentages. Where scanning is not performed and impressions given only by inspection of stained preparations, this induced error may not be so apparent as when attempts are made to afford quantitative evidence of abnormality. Nevertheless it constitutes an error which should be avoided if possible, especially in view of the fact that increasing tendency to obtain more fractions will multiply this error.

Where cellulose acetate membranes are used, albumin trailing still occurs, but the lowest trough obtained on scanning such strips is considerably shallower than that obtained in paper separations. The degree of trailing is apparently therefore much less, but experimental observation suggests that the degree of trailing may still be assessed by the ratio method mentioned above.

If the total protein is low, or if deliberate dilution is made prior to electrophoresis, similar low troughs may be encountered. The computation method, however, remains unchanged.

SUMMARY

The development of the albumin trail in paper protein electrophoresis has been considered using a variety of experimental states.

Using Whatman No. 1 paper and seeding specimens of volume 0.010 ml the trail is of the order of 9–14% of the total albumin in normal sera.

The albumin "carpet" may conveniently be assessed by drawing a horizontal

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line parallel to the abscissa of scan at a height above the abscissa equal to one third of the height of the minimal trough encountered in the trace of the stained strip. All the area between this line and the abscissa of scan is considered to be due to albumin, the area above this is attributed to the various globulin fractions. This approximation of the albumin trail seems to be a representative assessment whatever the conditions of electrophoresis.

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NOTE ADDED IN PROOF

A further examination of the method of prestaining using bromophenol blue, as indicated in the text of the article, has shown that in six further batches of the same dye, the absolute relationships were not reproducible. The fact that good recovery experiments were obtained, suggests however, that the manner of computing "albumin trailing", is not greatly in error. The conclusion arising from this observation, is that the particular batch of Bromophenol Blue must have contained some impurity which gave the approximate results which led to the development of the method. Two further methods, using a number of batches of dye, and linked with similar observations using Azocarmine B, have since been developed. These will be published in a later communication on Dyes and Dye-binding Capacity of Proteins. Meanwhile, it must be regarded that the method given in the text of the article of demonstrating albumin trail, is fortuitous, and cannot be repeated. The manner of calculation of the trail remains the same, and is supported by the new evidence which has been obtained.

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ULTRACENTRIFUGAL CHARACTERISTICS AND CARBOHYDRATE CONTENT OF MACROMOLECULAR γ-GLOBULINS*

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Normal human γ-globulin that is isolated electrophoretically is composed of two major ultracentrifugal components with sedimentation coefficients of 7 S and 19 S[1, 2]. Recently the 19 S fraction was shown to be relatively rich in hexose and hexosamine sugars and sialic acid with a total carbohydrate content approximately four times that of 7 S γ-globulin[3].

Globulins of high molecular weight occur as a major serum fraction in some rare disorders involving the reticulo-endothelial system. These pathological macroglobulins are closely related immunologically to the 19 S γ-globulin of normal serum[4, 5]. Several workers[6–10] have noted an intense staining reaction with periodic acid–Schiff following electrophoresis. Preliminary data from this laboratory on the hexose sugar content of the pathological macroglobulins[3] and quantitative periodic acid–Schiff analyses by LAURELL and associates[11] indicated a higher carbohydrate content as compared to the myeloma proteins.

The present paper reports in detail on the carbohydrate moiety of a number of isolated pathological macroglobulins and on some of their physical characteristics. It was found that they possess strikingly similar ultracentrifugal properties and that the carbohydrate content closely resembles that of normal 19 S γ-globulin, although some individual variations were encountered.

MATERIALS AND METHODS

Sera and macroglobulin preparations

Four macroglobulinemia sera (II, III, IV, and V) were furnished by different hospitals in the United States***, one (VI) was obtained from a hospital in Sweden. They were kept in the frozen state until they were subjected to preparative electrophoresis. None of the sera contained cryoglobulins. Isolation of the pathological fractions was accomplished by separating the sera by zone electrophoresis on polyvinyl chloride. The isolated proteins consisted to more than 95% of high molecular weight material. Only traces of 7 S-protein could be found in the purified preparations, except for macroglobulin MG IV that contained close to 10% of material with an *s*-rate of 7 S. MG I represents a crystalline macroglobulin that was isolated from the serum of a macroglobulinemia patient by KRATOCHVIL AND DEUTSCH[12]. Two fractions were prepared of MG III by differential ultracentrifugation, one with a relatively high, the other with a very low content of material with *s*-rates greater than 19 S.

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Electrophoresis

Preparative zone electrophoresis of sera was carried out employing as a supporting medium polyvinyl chloride, Resin 426, Goodrich Chemical Company, as described earlier[2]. Usually 10 ml of dialysed serum were applied to a block, measuring 40 × 26 × 1 cm. In some instances amounts up to 20 ml of serum were separated on a block without loss of resolution. In all experiments the potential gradient was 4–5 V/cm and the time approximately 36 h. Barbital buffer (pH 8.6, ionic strength 0.1) was used.

For the separation of neutral hexoses in protein hydrolysates a shorter polyvinyl block was employed with the dimensions 30 × 20 × 0.8 cm. Electrophoresis was performed in 0.05 *M* sodium borate, pH 9.2, at 4°, for 12 h, with a potential gradient of 8 V/cm. The material was applied 3 cm away from the cathode end of the block. The best resolution was observed when the concentration of each carbohydrate component in the test solution did not exceed 1 mg/ml, and when the initial band at the site of application was not wider than 0.5 cm. To achieve this, 2 ml of solution was brought into a slit of 6–8 cm length. For larger volumes longer slits were prepared. The width of the initial band could readily be estimated in cases where the test solution was colored. To colorless sugar solution one drop of phenolphthalein was added to render the initial band visible. The indicator color does not interfere with subsequent analyses, since it stays in the neighbourhood of the origin. Under these conditions mannose, galactose and glucose can be quantitatively separated from each other. Galactose and fucose also separate but do overlap. For analysis of the eluates obtained from 1 cm-wide segments the anthrone[13] and the cysteine method[14] were used.

Ultracentrifugation

For ultracentrifugal analysis a Spinco ultracentrifuge model E was used. The proteins were dissolved in barbital buffer, pH 8.6, ionic strength 0.1, and analysed at concentrations between 0.2 and 1%. The experiments were carried out at 20° at 52640 rev./min. The Schlieren patterns were evaluated according to TRAUTMAN[15]. To correct the observed *s*-rate of a given component of a macroglobulin for concentration dependence, the concentrations of itself and of the more slowly sedimenting components were taken into account. No correction was made for the Johnston–Ogston effect.

Preparative centrifugation of MG III was performed in a Spinco model L machine at 114,000 × *g* for 2 h. The protein concentration was 1%. A top and a bottom fraction were recovered.

Carbohydrate and protein determination

Hexose sugars were determined by the anthrone method according to MOKRASCH[13] and hexosamine according to BOAS[16], using adsorption to and elution from Dowex-50 to eliminate non-specific chromogens. The assay of fucose was performed as described by DISCHE AND SHETTLES[14] and of sialic acid using the method of WERNER AND ODIN[17]. Adaptation of these methods to eluates from the polyvinyl block, minor modifications, and standard deviations have been published earlier[2]. Protein was assayed by a modified Folin method[18]. Human serum albumin was used as reference substance.

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RESULTS

Zone electrophoretic and ultracentrifugal studies

The electrophoretic mobility of the macroglobulins as determined by zone electrophoresis on polyvinyl chloride varied from one protein to the other: protein MG V resembled normal β-globulin, MG II migrated between β- and γ-globulin, MG IV coincided with the peak of the γ-globulin fraction and MG III was still slower. At the pH employed for separation all four pathological proteins behaved as single, symmetrical components. Chemical homogeneity was indicated by the fact that hexose and protein concentrations closely parallelled each other throughout the area of the pathological fractions. Fig. 1 shows the electrophoretic pattern of macroglobulinemia serum V with the pathological fraction migrating as a β-globulin. Quantitative protein and hexose determinations on the eluates provide an impression of the distribution of protein-bound hexose. It is evident from this pattern that the hexose concentration in the pathologically elevated fraction is much higher than in the more slowly migrating γ-globulin.

On ultracentrifugal examination the isolated macroglobulins were found to be heterogeneous. All of them consisted of three heavy components, one major and two minor ones. Disregarding the small and varying amounts of 7 S material, the macroglobulins displayed a strikingly similar composition. The similarity, was fully disclosed only after extrapolation to infinite dilution, because the *s*-rates of the three heavy components showed a marked concentration dependence. Using the observed *s*-rates of the main component of MG II, III, and V, a *k*-value of 0.055 ml/mg was

TABLE I ULTRACENTRIFUGAL COMPOSITION OF DIFFERENT PURIFIED MACROGLOBULINS

Macroglobulin No.	$S^{\circ}_{20,w}$	*Per cent*
	19.0	76
II	27.8	17
	35.1	5
	18.8	71
III	31.0	21
	40.2	6
	19.1	76
IV	29.4	17
	37.9	5
	19.9	81
V	29.0	12
	39.8	6
	19.2	76.0
Mean	29.3	16.7
	38.2	5.5

ascertained[1]. The line of regression was found by least square analysis. Table I lists (for each component $S^{\circ}_{20,w}$) and the concentration in per cent of total macroglobulin protein. The main component of each of the isolated macroglobulins sedimented with an *s*-rate of approximately 19 S, the two minor components with *s*-rates of approximately 29 S and 35–40 S. The greater variation in the latter group is at least in part due to less accurate quantitation owing to the low concentration of the most rapidly sedimenting material. The physical similarity of the macroglobulins under investigation was underscored by the fact that the relative concentrations of their three components showed little variation from one case to the other.

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The ultracentrifugal composition of a given macroglobulin was not altered by changing environmental conditions. Identical patterns were observed at pH 1.5–9 and in 6 *M* urea.

The carbohydrate of macroglobulins

In view of the physical heterogeneity of the proteins under investigation the value of doing chemical analyses for further characterization seemed questionable. It is conceivable, for instance, that all the carbohydrate present in one preparation were attached to one of the minor ultracentrifugal components. To clarify this point, two fractions of different physical composition were prepared of MG III by differential ultracentrifugation. The top fraction contained predominantly 19 S material, while in the bottom fraction the 29 S and 38 S components were considerably enriched. Despite the marked physical difference of top and bottom fraction the hexose–peptide ratios were virtually identical for both fractions (Table II). This finding supports the view that the three components are very similar if not identical with respect to their carbohydrate content.

Table III shows the carbohydrate data of five isolated macroglobulins, one of which, MG I, represents a crystalline protein. For comparison the data on the 19 S γ-globulin of normal serum[3] are listed. All these proteins are very similar in hexose,

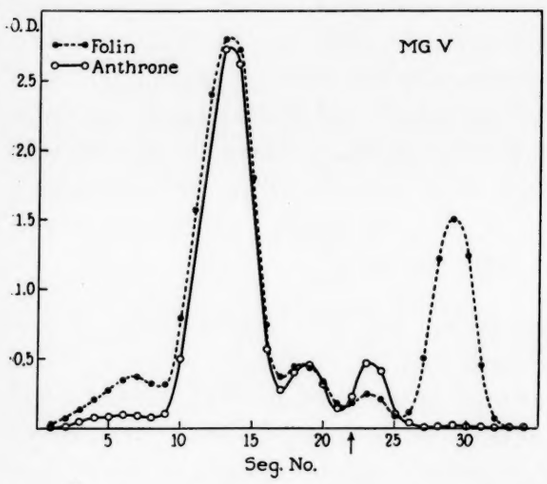

Fig. 1. Protein and hexose curves of macroglobulinemia serum V after electrophoretic separation on a polyvinyl chloride medium. The mobility of the abnormal fraction coincides with that of the β-globulin. The anthrone curve shows most of the protein-bound hexose associated with the pathological protein. The arrow indicates site of application.

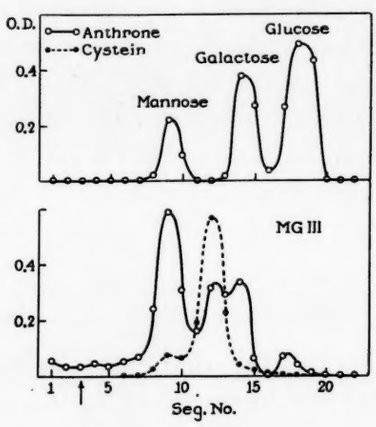

Fig. 2. Neutral hexose sugars of macroglobulin MG III hydrolysate separated electrophoretically on polyvinyl chloride using borate buffer (lower pattern). The anthrone curve shows three distinct peaks, one of which represents fucose as indicated by a positive cysteine reaction, the other two correspond to mannose and galactose of the standard solution (upper pattern).

TABLE II ULTRACENTRIFUGAL COMPOSITION AND CARBOHYDRATE CONTENT OF ULTRACENTRIFUGAL TOP AND BOTTOM FRACTION OF MACROGLOBULIN MG III

Macroglobulin III	*Ultracentrifugal composition*				$\frac{mg\ Hexose}{mg\ Peptide} \cdot 10^{-3}$
	% 7 S	% 19 S	% 29 S	% 38 S	
Ultracent. top	12	80	8	0	54
Ultracent. bottom	0	67	20	13	55

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TABLE III CARBOHYDRATE CONTENT OF DIFFERENT PATHOLOGICAL MACROGLOBULINS COMPARED TO NORMAL 19 S γ-GLOBULIN

Macro-globulin	$\frac{\text{mg Carbohydrate}}{\text{mg Protein}} \cdot 10^{-3}$				*Hexosamine / Hexose*
	Hexose	*Hexosamine*	*Fucose*	*Sialic acid*	
γ_1–19 S	61.6	33.1	7.4	20.1	0.55
I	59.0	29.9	7.5	20.7	0.55
V	57.2	31.9	7.1	17.7	0.56
II	59.2	29.6	7.3	18.7	0.50
IV	51.7	28.6	7.2	19.1	0.55
III	54.7	31.3	6.3	18.5	0.55

hexosamine, fucose and sialic acid content. The hexosamine–hexose ratio is almost uniformly 0.5. There are, however, some differences. The total carbohydrate content of the pathological macroglobulins tends to be somewhat lower than that of the normal heavy γ-globulin. Compared with normal 19 S γ-globulin MG IV contains 12%, MG III 8%, and MG I, II, and V 5% less carbohydrate.

To analyse further the neutral hexose portion of the carbohydrate moiety, hydrolysates of MG III and MG VI were studied electrophoretically employing borate buffer. Hydrolysates and standard solutions of hexose sugars were separated simultaneously on the same polyvinyl block (Fig. 2). Subsequent analysis with anthrone detected three peaks corresponding to mannose, galactose and fucose. The latter was identified by a strongly positive cysteine reaction and by comparison of its mobility with that of a fucose standard used in other experiments. The electrophoretic patterns of hexose sugars for MG III and MG VI looked essentially alike. The small elevation in the glucose region of the anthrone baseline present in the pattern of MG III was absent from that of MG VI. The relative concentration of the three hexose sugars was estimated on the basis of anthrone analysis. The following values were found for MG III: fucose 15%, galactose 20%, and mannose 65%.

DISCUSSION

The finding of highly similar ultracentrifugal properties for the pathological macroglobulins analysed in this study is in contrast to the considerable variation of *s*-rates reported in the literature for this type of protein. In their original description of macroglobulinemia, WALDENSTRÖM[19] and PEDERSEN AND WALDENSTRÖM[20] defined the pathological fraction as a 19–20 S protein. Others[21] have since reported *s*-rates ranging approximately from 13–21 S. It is difficult to decide whether the variations found in the literature reflect true molecular differences or rather differences of experimental conditions. In the majority of cases, macroglobulins have been examined without prior isolation from serum and the data reported were not corrected for concentration dependence. As the *s*-rate of macroglobulins is markedly concentration-dependent, this may, in fact, explain the scattering of the literature data.

On the other hand, pathological serum proteins occur which are definitely heavier than 7 S γ-globulin, and yet they must not be confused with macroglobulins proper. They have been found in the serum of myeloma patients and consist of multiple ultracentrifugal components of the 7–15 S variety. The heavier components of this type may be produced[2] *in vitro* at the expense of the 7 S-protein by lowering the pH.

The sedimentation coefficient of the main component of the four pathological

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proteins accords with that of the normal heavy γ-globulin[1]. The minor 29 S and 38 S components which also were observed by others[9, 10, 17, 22, 23], are not unique for pathological macroglobulins but seem to have their counterparts in the γ fraction of normal serum. Although their concentration in normal serum is too low to be detected, they may be rendered visible by enrichment using differential ultracentrifugation[1]. The fact that the carbohydrate content of a given macroglobulin preparation appeared to be independent of the amount of 29 S and 38 S material present, suggests that the latter may be related to the main component and that it was produced by polymerization. KORNGOLD[4], however, has obtained evidence of immunological differences between these different size macroglobulin components. Recent evidence[24, 25] that sulphydryl reagents depolymerize the minor and the major components of macroglobulins to 7 S-units throw light on the significant role of intramolecular S–S-bonds for these proteins.

Although composed of 7 S subunits, γ-globulins of high molecular weight differ chemically from ordinary 7 S γ-globulin, since they are particularly rich in carbohydrate. This has been demonstrated earlier for the normal 19 S γ-globulin[3] and is shown in this study to apply to the pathological γ-globulin occurring in macroglobulinemia WALDENSTRÖM[19]. The pathological proteins contained close to four times as much carbohydrate as 7 S γ-globulin and thus they bear strong resemblance to the normal 19 S γ-globulin. This is most apparent for the crystalline macroglobulin MG I and for MG II, and V, which differ from normal 19 S γ-globulin by no more than 5% in total carbohydrate. On the other hand there are definite deviations from the normal, the most obvious one being MG IV, with a total carbohydrate content that is 12% lower than that of normal 19 S γ-globulin. Whether the presence of 7 S protein in this preparation is partially responsible for the lower value, was not investigated. Similar investigations have previously been conducted on the carbohydrate moiety of isolated myeloma proteins[2]. Those of the γ type and normal 7 S γ-globulin were found to contain comparable amounts of carbohydrate, but in some cases significant deviations were detected. Regarding the carbohydrate moiety, the relationship between pathological macroglobulins and normal 19 S γ-globulin appears to be analogous. That the present series is relatively homogeneous with respect to carbohydrate content may in part be due to the fact that it is small and that therefore rare proteins with more striking deviations from the normal, if they occur, are not represented.

In a recent study which included carbohydrate analyses on six macroglobulinemia sera, LAURELL, LAURELL AND WALDENSTRÖM[11] estimated the hexose content of the macroglobulin fraction. The data were obtained by determining the difference in hexose content between a given macroglobulinemia serum and normal serum and dividing this by the protein concentration of the abnormal component as ascertained by paper electrophoresis. Using this method they found hexose values, roughly comparable to those reported in this paper, but showing considerably wider variation.

The views expressed in this paper, which are based on physical and chemical data, are in accord with results of immunological studies. It has been shown that 19 S γ-globulin and macroglobulins of the WALDENSTRÖM type are antigenically related[4, 5]. By the same methods it was revealed that normal and pathological γ-globulin of high molecular weight are antigenically completely unrelated to the 19 S α_2-globulin of normal serum[5].

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Attention has been called to the fact that certain biological activities are associated with 19 S γ-globulins. Thus activities attributed to the rheumatoid factor [26] and the cold-agglutinating activity [25] were shown recently to reside in the 19 S γ-globulin fraction of respective sera.

SUMMARY

Ultracentrifugal studies and carbohydrate analyses were carried out on a number of purified γ-globulins of high molecular weight, isolated from the serum of macroglobulinemia patients by preparative zone electrophoresis on polyvinyl chloride.

Four macroglobulins, studied ultracentrifugally, proved strikingly similar with respect to $S^\circ_{20,w}$ and the relative concentration of their three markedly concentration-dependent components. $S^\circ_{20,w}$ of the major component was 19 S and of the two minor ones approximately 29 and 38 S. Evidence was obtained that the three ultracentrifugal components do not differ markedly in carbohydrate content.

Five macroglobulins analysed for carbohydrates were found similar in hexose, hexosamine, fucose and sialic acid content. Containing close to four times as much total carbohydrate as normal 7 S γ-globulin, they resembled normal 19 S γ-globulin.

Two macroglobulins were analysed for individual neutral hexose sugars. Fucose, galactose, and-mannose were found in a relative concentration of approximately 15, 20 and 65%, respectively.

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THE ROLE OF THE BASIC FRACTION OF γ-GLOBULIN IN THE FLOCCULATION TESTS*

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Much evidence exists indicating that the thymol turbidity, cephalin flocculation and zinc turbidity tests reflect in part changes in the level and probably in the nature of the γ-globulin of serum[1-3]. It has been noted that γ-globulin from hepatitis serum reacts more strongly with thymol reagent and cephalin cholesterol antigen than normal γ-globulin[1]. Although these tests are most often abnormal in patients with hypergammaglobulinemia, occasional inconsistencies have been noted. These have been explained in part by changes in some of the other serum proteins, expecially the β-globulin fraction[4]. In addition, many observers have noted the potentiating role of lipids in the thymol turbidity test[5, 6] and the inhibitory action of albumin and possibly other serum fractions[7-9]. The zinc sulphate turbidity test reflects changes in the level of γ-globulin more specifically[3].

Techniques of zone electrophoresis were therefore applied in an effort to detect changes in the γ-globulin which might shed further light on the chemical basis of these tests and could help to explain some of the discrepancies noted in their clinical application. The main findings concern the importance of the basic fraction of the γ-globulin in the thymol turbidity and cephalin flocculation tests.

MATERIALS AND METHODS

Zone electrophoresis was carried out according to the methods described by KUNKEL[10] using potato starch as the supporting medium and barbital buffer pH 8.6, $\Gamma/2 = 0.05$ or 0.1.

Techniques of the thymol turbidity and zinc turbidity tests were similar to those previously described from this laboratory using barium sulfate as a standard[5]. The cephalin flocculation test was performed according to the method of HANGER[2] and was read immediately and after 2, 6 and 24 h. In the thymol turbidity test with isolated protein fractions, a small amount of lipoprotein isolated from normal human serum by ultracentrifugation at a solution density of 1.063 was added.

RESULTS

I. Reactivity of fractions of γ-globulin of different electrophoretic mobilities

Early in this study it was noted that the γ-globulins from a normal and a cirrhotic serum reacted equally well with cephalin cholesterol emulsion at all concentrations tested, whereas in the thymol turbidity test, γ-globulin from a cirrhotic serum was more active than normal γ-globulin. In an attempt to explain this observation,

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the reactivity of different subfractions of γ-globulin was determined. Fraction II γ-globulin and γ-globulins from 3 normal and 8 pathologic sera were separated by starch zone electrophoresis into 8–10 fractions of different electrophoretic mobilities.

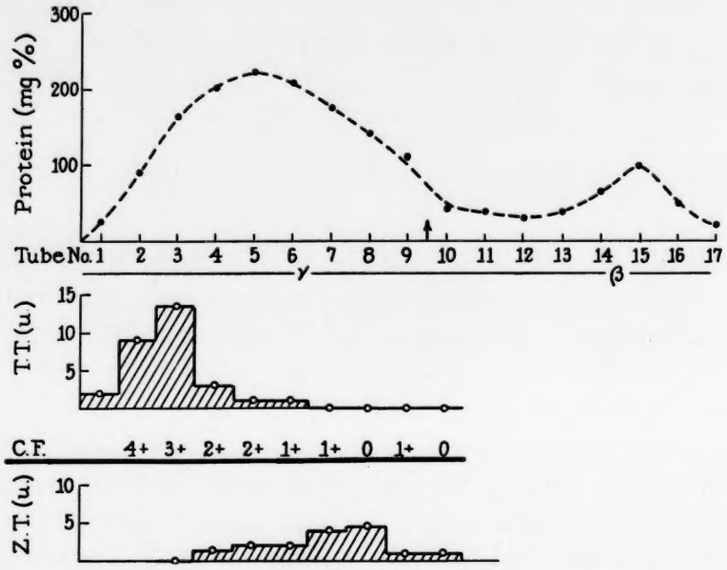

Fig. 1. Relative reactivities of subfractions of γ-globulin from a patient with cirrhosis of the liver in the thymol turbidity (T.T.), cephalin flocculation (C.F.) and zinc sulphate turbidity (Z.T.) tests.

Each of these was then adjusted to the same protein concentration and tested in the three flocculation reactions. The activities of equal amounts of fractions with different electrophoretic mobilities from a patient with cirrhosis are shown in Fig. 1. In the thymol turbidity and cephalin flocculation test the basic γ-globulins migrating more

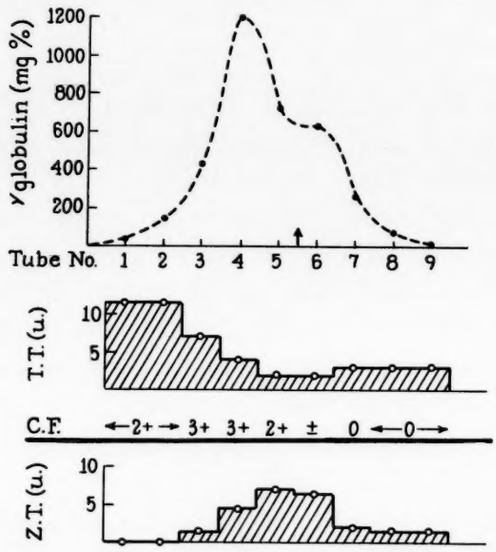

Fig. 2. Relative reactivities of subfractions of Fr. II γ-globulin in the thymol turbidity (T.T.), cephalin flocculation (C.F.) and zinc sulphate turbidity (Z.T.) tests.

slowly than the peak of the curve reacted most vigorously, while with zinc sulphate, fractions of intermediate mobility located under the peak and immediately adjacent to it were most active. Fig. 2 illustrates the results obtained with γ-globulins of various mobilities prepared from Fraction II γ-globulin. Here again the basic fractions reacted

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most vigorously with thymol reagent and cephalin cholesterol antigen. Similar results were obtained with γ-globulins prepared from 3 normal and 7 pathological sera. In all of these, the basic fractions of γ-globulin reacted more strongly than the more negatively charged molecules in the thymol turbidity and cephalin flocculation tests. The only exception was noted with γ-globulin prepared from a patient with infectious hepatitis where the most rapidly migrating fractions were again active.

II. The role of the electric charge of proteins in the turbidity tests

Since these experiments showed that the more basic components of γ-globulin were most active in causing flocculation of the cephalin cholesterol emulsion and turbidity with thymol reagent, an effort was made to evaluate further the importance of the electric charge of the proteins in determining their capacity to react with the flocculating reagents. Several proteins with different isoelectric points normally present in serum, and others not found in serum were tested. As previously noted by others[1, 3, 11], γ-globulin was the only serum protein to react in these tests. None of the other serum proteins such as albumin, α_2- and β-globulin, all of which are more acid than γ-globulin resulted in positive tests. Table I lists several other proteins with

TABLE I

THE ACTIVITY OF PROTEINS WITH DIFFERENT ISOELECTRIC POINTS IN THE FLOCCULATION TESTS

Protein (700 mg%)	*Isoelectric point*	*Thymol turb. (units)*	*Zinc turb. (units)*	*Cephalin flocc.*		
				Imm.	*6h*	*24 h*
Egg albumin	4.6	0	0	0	0	0
Human albumin	4.7	0	0	0	0	0
β-Lactoglobulin	5.2	0	0	0	0	0
Hemoglobin A	6.9	5.5	9	0	0	2+
γ-Globulin	6.0–8.5	4.5	10	0	2+	4+
Ribonuclease	9.5	4.5	0	0	4+	4+
Trypsin	10.3	22	0	2+	4+	4+
Lysozyme	10.5	29	0	2+	4+	4+

isoelectric points between 4.7 and 10.5 and their reactivities in each of the tests. The most acid ones (egg albumin and β-lactoglobulin) failed to react with thymol reagent and cephalin cholesterol antigen. Lysozyme and trypsin were most basic and reacted most vigorously in the thymol turbidity and cephalin flocculation tests, while γ-globulin, ribonuclease and hemoglobin occupied intermediate positions in reactivity and basicity. None of the proteins reacted with thymol reagent in the complete absence of lipids. In the zinc turbidity test only γ-globulin and hemoglobin A were active.

III. Distribution of γ-globulin in sera

In order to determine whether these observations might explain abnormalities of the flocculation tests in whole sera, groups of 6–9 sera with normal and abnormal flocculation tests were separated simultaneously by starch zone electrophoresis and the relative and absolute concentrations of the various subfractions of the γ-globulin were determined. For purposes of these experiments γ-globulin was divided into two main parts: a basic fraction migrating more slowly than the peak of the γ-globulin curve which reacted more strongly with thymol reagent and cephalin cholesterol

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antigen, and a more rapidly migrating part including the peak of the γ-globulin curve and the area immediately adjacent to it.

In sera from patients with cirrhosis of the liver, rheumatoid arthritis, lupus erythematosus, sarcoidosis and a number of other diseases, positive thymol turbidity and cephalin flocculation tests were usually associated with broad flat γ-globulin curves characterized by a relative and absolute increase in the basic fraction. However, elevated values in the thymol turbidity test were frequently noted in the absence of these changes in patients with infectious hepatitis whose sera contained large amounts of lipid. Occasional discrepancies were also noted in sera from patients with multiple myeloma and macroglobulinemia of Waldenström. However, in the majority of these sera an elevated thymol turbidity test indicated that the abnormal protein had the mobility of a slow γ-globulin, while a high zinc turbidity suggested the presence of a γ-globulin of intermediate mobility. Both tests were often negative in sera with high protein concentration if the paraprotein had the mobility of a fast γ- or β-globulin.

DISCUSSION

The importance of the γ-globulin fraction of serum in the thymol turbidity, cephalin flocculation and zinc turbidity reactions has been recognized since the introduction of these tests. MACLAGAN[12] analyzed the precipitate obtained in the thymol turbidity reaction and found it to consist of about 40% protein. Because of its low solubility he suggested that it was probably γ-globulin.

Qualitative as well as quantitative changes in the γ-globulin have been implicated. Thus MACLAGAN AND BUNN[1] noted that while γ-globulins separated from hepatitis sera caused flocculation with thymol reagent, the protein from normal sera resulted only in turbidity but no flocculation. They found that γ-globulin derived from hepatitis serum reacted somewhat more readily with thymol reagent and cephalin cholesterol antigen than normal γ-globulin. ALBERTSEN *et al.*[11] found the reverse to be true for the thymol turbidity test. MARTIN[13] attributed positive tests in certain instances to the presence of increased amounts of high molecular weight proteins which are normally present in sera. Some observers[4] have questioned the importance of the γ-globulin fraction and found that thymol reagent precipitated primarily β-globulin. The role of the serum lipids in addition to the γ-globulin in the thymol turbidity test was emphasized by KUNKEL AND HOAGLAND[5] and the inhibitory effect of albumin, especially when derived from normal sera[1] and of serum mucoproteins[8] was noted by MACLAGAN *et al.*

The changes responsible for positive results in the cephalin flocculation test are even less certain. HANGER and co-workers[6, 14] have shown that γ-globulin alone can cause flocculation of the cephalin cholesterol emulsion and HANGER[9] has postulated the presence in normal sera of a labile lipid-rich constituent of the albumin α_1-globulin fraction which inhibits this reaction. According to this observer[9] positive tests may be caused either by the disappearance of this factor or by changes in the reactivity of the γ-globulin.

Our studies have confirmed some of these observations and extended others. The important role of the γ-globulin has again been demonstrated. In addition, it has been shown that the fractions of γ-globulin that are most basic in charge reacted more strongly with thymol reagent and cephalin cholesterol emulsion, while only the γ-globulin of intermediate mobility was precipitated by zinc sulphate. The observations

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that the more acidic proteins failed to react and that basic proteins such as lysozyme, trypsin and ribonuclease were readily precipitated by thymol reagent and cephalin cholesterol emulsion, further emphasize the importance of the electric charge in these reactions. Of all the proteins tested only γ-globulin and hemoglobin reacted with zinc sulphate.

A possible explanation for these findings may be found by examining the physicochemical nature of protein solutions[15]. Most protein solutions consist of colloidal particles whose continuous kinetic motion brings them closely together, thus causing them to precipitate. The particles remain in the form of a stable solution only as long as the forces keeping them apart are able to overcome the attracting forces. In lyophobic colloidal systems stability is insured by the presence of a double layer of ions in the region of the interface, while in lyophilic colloids the added factor of a layer of solvation assumes importance in preventing flocculation. Any factor that reduces the potential across the double layer will decrease the stability of the solution and will, therefore, create optimal conditions for precipitation in the presence of flocculating reagents. Since cephalin cholesterol emulsion is usually negatively charged in relation to water, flocculation occurs most readily with more basic substances that tend to neutralize the charge on the colloidal particles. A similar mechanism may be operative in the thymol turbidity test in which thymol by virtue of its slightly negative charge may reduce the charge density of the more basic proteins thereby making them unstable. In the zinc sulphate turbidity test, the reaction is carried out near the isoelectric point of γ-globulin, thus resulting in selective precipitation of the γ-globulin by this protein-precipitating agent.

The clinical importance of these results on isolated protein fractions is born out by the observation that in sera with elevated thymol turbidity and positive cephalin flocculation tests there was usually a relative and absolute rise in the basic fraction of γ-globulin. However, exceptions were frequently noted in sera from patients with hepatitis associated with high serum lipids and occasionally in multiple myeloma and macroglobulinemia. Sera with positive zinc turbidity tests usually contained increased amounts of γ-globulin of intermediate mobility. The importance of the basic fraction of γ-globulin in these tests has recently been borne out in a comprehensive study on the flocculation reactions by ADNER[16]. In a group of sera from patients with multiple myeloma MANDEMA[17] also noted some relationship between the electrophoretic mobility of the protein and these turbidity tests.

While selective changes in the basic fraction of the γ-globulin may explain some of the discrepancies noted in the clinical applications of these tests, many others remain to be explained. Some of these are probably due to elevations of serum lipids which are known to potentiate the thymol turbidity test. Other inconsistencies remain and will have to await the study of isolated proteins other than γ-globulin and albumin, some of which may exert inhibitory effects on these reactions.

Although it is not possible on the basis of these studies to decide conclusively whether these tests reflect merely an increase in the γ-globulins normally present or the existence of abnormal proteins, recent studies favor the view that in the majority of cases, with the possible exception of certain paraproteinemias, they are caused by an increase in one of the fractions of γ-globulin present in normal serum. Ultracentrifugal analysis of γ-globulins separated from sera with positive flocculation tests showed only the presence of the normal 7 S and 19 S components[18]. There was usually

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a marked increase of the amount of γ-globulin which was primarily due to an elevation of the major 7 S component. It therefore seems possible that the 19 S fraction does not play a major role in the flocculation reactions as suggested by MARTIN[13] and more recently HARTMANN, GRABAR and co-workers[19]. Similarly, immunologic studies using the agar diffusion techniques also failed to demonstrate any qualitative differences between γ-globulins derived from normal sera and those prepared from patients with liver disease whose sera reacted strongly in the flocculation tests[20].

SUMMARY

1. Subfractions of γ-globulin of different electrophoretic mobilities reacted differently in the turbidity tests. The most basic fraction was most active in the thymol turbidity and cephalin flocculation tests and the intermediate fraction under the peak reacted strongly with zinc sulphate.

2. Study of other proteins of varying isoelectric points also showed that the more basically charged proteins reacted most strongly with thymol reagent and cephalin cholesterol antigen.

3. Examination of a group of normal and pathologic sera with abnormal flocculation tests showed some correlation between the level of the basic fractions of the γ-globulin and positive cephalin flocculation and thymol turbidity tests. However, exceptions were noted particularly in sera of patients with infectious hepatitis and multiple myeloma. The level of zinc turbidity was related somewhat more closely to the total level of γ-globulin.

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URINARY EXCRETION OF FREE AMINO ACIDS IN NORMAL ADULT MEN AND WOMEN

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Amino-aciduria has been investigated in many pathological conditions. Yet our present understanding of the actual findings and of the physiological processes involved remains unsatisfactory in many ways because we lack sufficiently precise quantitative knowledge of the daily excretion of each of the amino acids in urine, and also of their plasma levels. Until this has been more precisely assessed, the prospects of our understanding amino-acid excretion are bound to remain poor.

The object of the present paper is to make a contribution to the study of the first of those two factors: normal amino-acid excretion, irrespective of diet habits. It is further restricted to the output of *free* amino acids from only healthy *adults* of both sexes. This limitation is imposed by the facts that during growth the situation is not necessarily comparable to the condition in adults, that in early infancy the situation is known to be different, and, moreover, that independently of age, the kidney also excretes at least certain amino acids in combined form. Further investigation regarding these various factors will be needed in the future.

In the past, paper chromatography has predominantly contributed, and most usefully, to studies on amino-aciduria in disease. That it has made such a major contribution to the subject is chiefly because it led to a convenient and rapid procedure. There is no doubt however, that progress in our knowledge can hardly be expected if further steps do not involve more accurate quantitative data, even if the more elaborate column chromatographic methods which are required for the purpose are unfortunately so much more time- and labour-consuming. Nevertheless MOORE AND STEIN'S methods [1, 2] using ion exchange columns have undoubtedly opened the way to the required progress and it is to be anticipated that MOORE, SPACKMAN AND STEIN'S completely automatic procedure [3] will bring about a very great improvement compared with their original methods, as far as speed and convenience are concerned.

Prior to our own observations STEIN'S data [4] and those of EVERED [5, 6] formed the major contributions to our present subject. They used MOORE AND STEIN'S original procedure [1], whereas we have used their more recent alternative technique [2] which is to be considered as definitely more appropriate for urine analysis (see below). Certain technical differences have been adopted in our laboratory which have already been described (SCHRAM [7], SCHRAM *et al.* [8]).

EXPERIMENTAL

In Table I we compute the various findings as observed in healthy males by STEIN [4], EVERED [5, 6], and ourselves, and Table II refers, in the same way to healthy

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TABLE I

URINARY EXCRETION OF FREE AMINO ACIDS IN NORMAL HEALTHY MALES
(mg/24 h-collection)

Amino acid	*Range*			*Average values*		
	Stein [a] *6 cases*	*Evered* [a] *3 cases*	*Soupart* [b] *6 cases*	*Stein*	*Evered*	*Soupart*
Ornithine	–	–	0–4 [g]	–	–	1
Arginine	–	–	0–14	<10	–	6
α-Aminoadipic acid	–	–	5–13	–	–	8
Methionine	–	5–9	5–11	<10	6	7
Aspartic acid	–	–	3–29	<10	–	8
β-Alanine	–	–	3–10	–	–	6
Valine	4–10	5–8	4–17	<10	5	10
Lysine	7–48	7–17	0–14 [g]	19	12	7
Cystine	10–21	5–15	3–33	<10	9	14
Leucine	10–25	5–10	6–20	14	8	11
Isoleucine	10–30	5–22	8–24	18	13	15
Phenyl-alanine	9–31	11–18	8–15	18	14	13
Tyrosine	15–50	18–26	7–27	35	23	19
Threonine	15–50	17–37	2–35	28	28	17
β-Aminoisobutyric acid	–	9–18	6–37	–	13	22
Alanine	20–70 [d]	17—37 [d]	5–32	46	28	22
3-Methyl-histidine	–	33–47 [e]	35–87 [g, h]	–	40	65
Serine	25–75	44–134	27–65	43	93	42
Glutamine [c]	40–100	–	42–103	–	–	73
1-Methyl-histidine	50–210	9–29	22–114 [g]	180	22	73
Histidine	110–320 [f]	59–130 [f]	20–213 [g]	216	97	138
Taurine	85–300	35–81	44–231	156	59	123
Glycine	70–200	69–148	53–189	132	109	104

For footnotes see under Table II.

females, the data being restricted to one case studied by EVERED and our present findings in 9 female subjects.

The diets were not controlled. In the case of female urine, the 24-h collections were made between the 14th and the 20th day of the menstrual cycle, because it has been shown, at least for histidine, that there is a maximum urinary output during this period (SOUPART[10]).

The amino acids are listed according to increasing order of average excretion values and not according to elution order. This has been found more convenient since the elution order varies with the type of chromatographic method used.

In Table III, our personal data for healthy males and females are reproduced in amino acid equivalents in order to give a better account of the relative amounts of free amino acids excreted by normal subjects. These findings are visualized in Fig. 1.

Examination of Table I and II shows clearly the improvement resulting from the use of the modified MOORE AND STEIN method[2].

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TABLE II

URINARY EXCRETION OF FREE AMINO ACIDS IN NORMAL HEALTHY FEMALES
(mg/24 h-collection)

Amino acid	*Evered: 1 case* [a]	*Soupart: 9 cases* [b]	
	Single value	*Range*	*Average*
Ornithine	–	0–11 [g]	2
Arginine	24	0–11	4
α-Aminoadipic acid	–	0–13	4
Methionine	5	3–12	5
Aspartic acid	–	2–11	4
β-Alanine	–	2–9	3
Valine	5	0–30	6
Lysine	8	0–16 [g]	8
Cystine	5	0–13	6
Leucine	5	2–16	9
Isoleucine	5	5–20	10
Phenyl-alanine	9	6–41	13
Tyrosine	11	9–26	15
Threonine	11	5–33	23
β-Aminoisobutyric acid	5	10–52	29
Alanine	12 [d]	9–44	24
3-Methyl-histidine	21 [e]	30–69 [g, h]	48
Serine	30	22–61	37
Glutamine [c]	–	43–88	62
1-Methyl-histidine	10	26–155 [g]	65
Histidine	130 [f]	79–208 [g]	128
Taurine	80	27–161	87
Glycine	24	67–312	142

[a] 100-cm column of Dowex 50-X8 or X12 (MOORE AND STEIN[1]).

[b] 150-cm column of Dowex 50-X5, elution gradient of gradually increasing pH and concentration (MOORE AND STEIN[2]).

[c] Contains glutamine, asparagine, sarcosine, calculated as glutamine. Glutamic acid is also included and added on molar basis, since no glutamic acid is found in freshly voided urine (STEIN[4]). Moreover glutamine is not recovered quantitatively from Dowex 50-X5 150-cm column.

[d] Includes α-aminoadipic acid (100-cm column).

[e] Values obtained on 15-cm column, which has a poor resolving power in this range in the case of urine.

[f] Histidine peak obtained on 100-cm column contains 3-methyl-histidine. These results, although minimal owing to incomplete recovery of the basic amino acids in experiments of this type, involve an overestimation for histidine.

[g] Are to be considered as maximal values because of elevated blank reading in the range of the NH_3 peak to the 3-methyl-histidine peak, (MOORE AND STEIN[2]). For histidine, values obtained by an enzymic specific decarboxylation method are a little lower than those obtained on 150-cm column (SOUPART[9]).

[h] May include some anserine and carnosine, if present in urine.

[i] Contains asparagine and glutamine, calculated as serine.

– Implies that no range is given by authors.

o Implies that, at least in one of the cases investigated, no peak was found for this amino acid. The arithmetic mean was thus calculated including the number of cases where no peak was found.

Note: Not included in SOUPART's data are citrulline, which is part of the glycine peak, and γ-aminobutyric acid, traces of which seem to be present in almost all cases, but has not been definitely identified by use of the usual criteria (STEIN[4]). Tryptophan, not recovered quantitatively on Dowex 50, is not included.

Values lower than 10 mg/24 h are not to be taken as absolute.

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TABLE III

FREE URINARY AMINO ACIDS OF HEALTHY ADULTS

(m$M \cdot 10^{-2}$/24 h-collection)

Amino acid	*Women*			*Men*			*General range for both sexes*	*Average*	*Average in % of total average figures*
	Lower	*Higher*	*Average*	*Lower*	*Higher*	*Average*			
Ornithine	0	8	1	0	3	0.5	0–8 [a]	1.1	0.17
Arginine	1	6	2	2	8	3	1–8	2.6	0.41
α-Aminoadipic acid	0	8	3	3	8	5	0–8 [a]	3.4	0.54
Methionine	2	8	3	2	7	5	2–8	3.6	0.57
Aspartic acid	1	8	3	1	22	6	1–22	4.1	0.65
β-Alanine	2	10	3	4	12	6	2–12	4.7	0.74
Valine	0	26	3	4	15	9	0–26 [a]	7.2	1.13
Lysine	0	12	4	0	10	5	0–12 [a]	4.4	0.69
1/2 Cystine	3	11	6	3	28	12	3–28	8.2	1.29
Leucine	1	12	7	2	15	8	1–15	7.4	1.17
Isoleucine	4	15	7	6	18	11	4–18	9.4	1.48
Phenyl-alanine	4	25	8	5	9	8	4–25	8.0	1.26
Tyrosine	5	15	9	4	15	11	4–15	9.4	1.48
Threonine	4	27	19	2	30	15	2–30	16.8	2.65
β-Aminoisobuturic acid	0	51	25	6	36	22	0–51 [a]	25.2	3.97
Alanine	10	50	26	6	36	24	6–50	25.7	4.05
3-Methyl-histidine	18	41	28	21	52	38	18–52	32.3	5.09
Serine	21	58	36	26	62	40	21–62	37.4	5.89
Glutamine	30	53	42	29	77	50	29–77	51.5	8.11
1-Methyl-histidine	16	93	43	13	67	43	13–93	43.3	6.82
Histidine	13	134	74 [b]	13	137	90 [b]	13–137	79.0	12.45
Taurine	22	129	70	35	185	99	22–185	81.2	12.80
Glycine	89	416	190	71	252	138	71–416	168.7	26.58

[a] Ornithine was found in 4 out of 15 cases studied, α-aminoadipic acid in 13 out of 15, valine in 11 out of 13 (2 determinations lost), lysine in 10 out of 14 (1 determination abnormally high not included), β-aminoisobutyric acid in 14 out of 15.

[b] Average of 27 female subjects and of 11 male subjects.

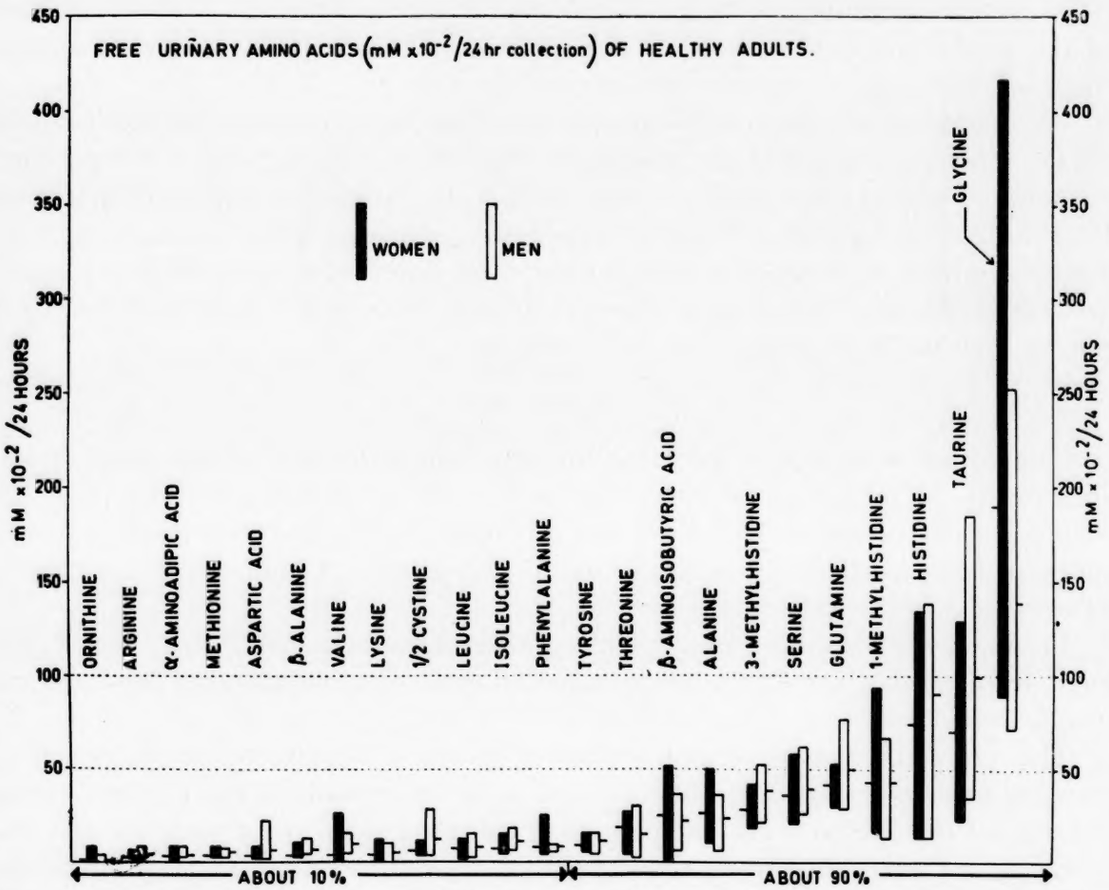

Fig. 1. Free urinary amino acids of healthy adults. m$M \cdot 10^{-2}/24$ h-collection. The marks on the side of each range indicate the average values. For amino acids listed from ornithine to phenylalanine, the output is so small that the average values calculated are not to be taken as being significant. The bulk excretion of those 12 amino acids amounts to less than 10% of total excretion.

There is an increased resolving power associated with a greater ease of operation and better quantitative recoveries for the basic amino acids. The increased resolving power is of special importance with regard to the separation of histidine, 3-methyl-histidine and 1-methyl-histidine. It is also particularly important in the range of elution of aspartic acid, threonine, serine and glutamine.

As glutamic acid is absent from freshly voided urine (STEIN [4]), the glutamic acid found is the result of glutamine decomposition during collection and storage of urine, so the glutamic acid has been added, on a molar basis, to the glutamine peak and calculated as such. Furthermore, it must be borne in mind that the glutamine peak obtained by the modified technique [2], also contains asparagine and sarcosine. The values given for glutamine are liable, therefore, to be somewhat too high.

When considering the individual range of excretion it can be seen that the data listed in Table I and II agree well in general. The most significant difference is for histidine. This is due to the fact that histidine and 3-methyl-histidine are not separated on the 100-cm column and are poorly separated on the 15-cm column of Dowex 50-X8 or X12. Our personal histidine determinations have been controlled by an specific enzymic decarboxylation method (SOUPART [9]) and the values obtained were only a few percent lower than those obtained on a 150-cm column of Dowex 50-X5.

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This is easily understandable owing to the greater specificity of the enzymic method and also to the fact that there is an elevated blank reading in the range of elution of histidine on a 150-cm column (MOORE AND STEIN [2]).

Two amino acids known to be present in normal urine are not included in these tables: γ-aminobutyric acid and tryptophan. The former, when a trace of it is present, is excreted at a level not exceding a few mg/24 h. In our cases it is present in almost all the urines investigated, but by the usual criteria, the suspected peak has not been actually identified as being γ-aminobutyric acid, the amount being too small. Although traces of tryptophan are always present, in experiments of this kind recovery is poor, so it has not been included in any of the data.

DISCUSSION

Comparison with values from the literature for individual amino acids, determined mostly by microbiological methods, will not be attempted here. This point has been excellently reviewed by STEIN [4] and furthermore, the main objection to microbiological data, which is the microbiological availability of amino-acid derivatives and combined forms, still remains uncleared.

Proline, hydroxyproline, glucosamine and lanthionine have regularly been found absent from normal urine. Hydroxylysine and ethanolamine have not been systematically looked for.

The use of the 150-cm column of Dowex 50-X4 or X5 enables one to recognize in normal urine about 40 different ninhydrin-positive substances, 29 of them forming the group of free amino acids. We include in this group taurine, asparagine and glutamine. Under these experimental conditions ninhydrin-reacting peptides containing up to 8 or 10 amino-acid residues should show up on the chromatograms as well-defined peaks if their color factor allows for it. The remaining unidentified peaks (about a dozen of them) correspond to unknown amino acids or to amino-acid derivatives or to peptides. They appear as very small peaks either because they are excreted in minute amounts or because their color factor is very low. Many of them are acid-stable. Acid hydrolysis of the urine samples produces a marked increase in some of the amino acids of the group of identified substances and a minor increase of almost all the other amino acids of the same group. The bulk excretion of combined amino acids is about twice that of the free amino acids (STEIN [4]). This group of combined amino acids is chiefly composed of substances such as hippuric acid or phenylacetyl-glutamine and even some peptides although the presence of the latter in urine is still a matter for debate. WESTALL [11] and BOULANGER *et al.* [12] claim that there is evidence of the presence of peptides in urine. The term "combined amino acids" refers in our opinion to substances in which the amino acid is linked to another substance by its amino groups. We therefore include compounds such as glutamine and asparagine or still others, such as tyrosine-O-sulphate (TALLAN [13]), in the group of free amino acids. The expression amino-aciduria is used in this paper to refer to free amino-acid excretion. Comparison of the data obtained from normal healthy men and women, and examination of Fig. 1, shows that no gross sex-linked difference between the general excretion pattern of amino acids can be recognized, although a sex-induced regulation exists which controls the excretion of some of the amino acids in female subjects during the menstrual and reproductive cycle (SOUPART [10]). For the purpose of studying amino-aciduria, measurement of the total excretion of free amino

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acids does not give sufficient information. In the present study, values found for the total amino-acid excretion and calculated from the column data, ranged from 0.48–1.11 g2/4 h in females and from 0.35–1.18 g/24 h in males, the average figures being 0.73 and 0.80 g respectively. The average figure found by STEIN [4] for men is 1.1 g/24 h.

When amino-acid excretion is calculated from the present data in terms of α-amino nitrogen, it appears that values range from 59–132 mg/24 h in females and from 41–133 mg/24 h in males. The average figures are 87 and 91 mg/24 h, respectively, of α-amino-nitrogen. These results seem in fairly good agreement with the value of 1% of total nitrogen, usually given in the literature, the latter being excreted at levels varying between about 8 and 18 g daily. Among the 23 substances listed in Table III and usually found in normal adult urine, 22 are free amino acids and the 23rd an amino-acid derivative, taurine, which ranks second after glycine in decreasing order of excretion. The average outputs of free amino acids may be listed as follows, in % of the total amount, on a molar basis:

Glycine	27%	Serine	6%
Taurine	13%	3-Methyl-histidine	5%
Histidine	12%	Alanine	4%
Glutamine	8%	α-Aminoisobutyric acid	4%
1-Methyl-histidine	7%	Threonine	3%

These 10 amino acids amount to 89% of the total output of free amino acids, the remaining 13 amino acids forming only 11% of the total. Differences in the average figures of the latter are therefore not to be taken as significant.

SUMMARY

The daily excretion in human urine of 22 free amino acids and of taurine, has been studied in six healthy adult males and nine females and the data compared to those previously reported in the literature. The chromatographic method used is that of MOORE AND STEIN with Dowex 50-X5 and elution gradient.

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DISSOCIATION OF A HUMAN SERUM MACROGLOBULIN IN ACID BUFFER

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Macroglobulins are defined as serum proteins which have an uncorrected sedimentation constant in the ultracentrifuge of 15 S or greater. Normal human serum presents a small 12–15 S peak[1]; however, in macroglobulinemia, a rare syndrome, a large amount of serum protein with a sedimentation constant of 15 S or greater is found. This syndrome, first recognized by WALDENSTRÖM[2], is also frequently accompanied by a bleeding tendency, increased blood viscosity, and the presence of an abnormal cell in the bone marrow. The electrophoretic mobilities of these proteins are generally in the γ- or β-globulin regions. Recent immunological studies[3] have shown these proteins to be abnormal in that they (1) lack several normal antigenic groups and (2) contain other antigenic groups that are patient-specific. These macroglobulins do, however, have some antigenic groups in common with the macroglobulin and 7 S γ-globulin of normal serum.

PEDERSON[4] suggested that macroglobulins may be aggregates of smaller globulin molecules. PETERMANN AND BRAUNSTEINER[5] later presented evidence indicating that conditions leading to the dissociation of aggregated molecules, such as dilution and increased temperature, reduced the relative concentration of the heavier species. DEUTSCH AND MORTON[6] dissociated a macroglobulin into 6.5 S components by use of cysteine or mercaptoethanol which break covalent disulfide bonds. Removal of these reagents by dialysis resulted in re-aggregation of the 6.5 S components into molecules whose sedimentation pattern differed from that of the parent macroglobulin. FRANKLIN *et al.*[7] were able to dissociate a 22 S protein present in the serum of a patient with rheumatoid arthritis into nearly equal amounts of 19 S and 7 S components with either urea or acid pH. However, the dissociation of a macroglobulin

TABLE I

THE SEDIMENTATION CONSTANTS AND CONCENTRATIONS

Expt.	*Preparation*	*Buffer*	*4 S*		
			$S^o_{20,w}$	$S_{20,w}$	*Conc. mg/ml*
A	serum	Barbital pH 8.62	5.3	3.9	15.4
B	serum	Acetate pH 4.25	4.7	4.2	7.4
C	γ-globulin fraction	Barbital pH 8.62	—	—	—
D	γ-globulin fraction	Acetate pH 4.25	—	—	—
E	γ-globulin fraction	Barbital pH 8.62	—	—	—

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at acid pH has not been generally observed[6, 8]. A macroglobulin showing a striking degree of dissociation in acid buffer is described below.

EXPERIMENTAL

Serum was obtained from a patient at Walter Reed Army Hospital with the clinical symptoms of macroglobulinemia. The electrophoretic pattern indicated that the γ-globulin concentration was greatly increased. The serum was prepared for study either (1) by dilution with buffer and exhaustive dialysis at 4°, or (2) by fractionation of γ-globulin by dialysis (4°) against 1.6 *M* ammonium sulfate in phosphate buffer[9] at pH 7.0 and subsequent dissolution of the precipitate in the desired buffer and dialysis. Two buffers were used: barbital $\Gamma/2$ 0.1, pH 8.62 and acetate $\Gamma/2$ 0.1, pH 4.25. One aliquot of the γ-globulin fraction (experiment E) was dissolved in and dialysed against the acetate buffer for 10 hours, and then dialysed exhaustively against several changes of the barbital buffer in order to test the reversibility of the acid dissociation. The γ-globulin fraction was free of α- and β-globulin within the limits of detection by paper electrophoresis.

The centrifugation was performed in the Spinco Model E ultracentrifuge equipped with a phase plate. A 12-mm analytical cell was used. The temperature of each run was considered to be the mean of the rotor temperatures at the beginning and end of the run corrected[10] by —0.9° for the temperature effect discovered by WAUGH AND YPHANTIS. Sedimentation constants were calculated from measurements on enlarged tracings; and when peaks of major components were not completely resolved, Gaussian curves were constructed and the maxima of these peaks were used. Corrections of $S_{20,w}$ were made in the usual manner for temperature, density, and viscosity, using a $\bar{v}$ value of 0.73. $S^\circ_{20,w}$ was calculated by the method of WALLENIUS, *et al.*[1] according to the formula $S_i^\circ = S_i^{\circ\,obs}/(1 - \sum_1^i k_j C_j)$ where C_j is the concentration and $k_j = 0.007$ ml/mg for all components except those with $j = 19$ S or 28 S for which $k_j = 0.017$ ml/mg. The concentration of each component was obtained by determining the area under each peak with a Maho planimeter and applying the formula of PICKELS[11] using $\triangle n$ as 0.00188. The concentrations thus derived were

OF COMPONENTS OF A HUMAN SERUM MACROGLOBULIN

Component											
7 S			*12 S*			*19 S*			*28 S*		
$S^o_{20,w}$	$S_{20,w}$	*Conc. mg/ml*	$S^o_{20,w}$	$S_{20,w}$	*Conc. mg/ml*	$S^o_{20,w}$	$S_{20,w}$	*Conc. mg/ml*	$S^o_{20,w}$	$S_{20,w}$	*Conc. mg/ml*
8.1	6.0	5.0	12.7	9.5	4.3	19.5	14.5	3.7	26.9	20.1	1.3
6.9	6.1	6.8	—	—	—	18.5	16.4	0.8	—	—	—
7.5	6.5	4.3	11.9	10.4	4.2	19.8	17.2	3.7	28.4	24.7	0.9
6.5	6.0	10.7	—	—	—	19.7	18.2	0.4	—	—	—
7.5	6.7	4.7	12.4	11.1	2.4	15.5	13.9	3.1	28.7	25.7	0.3

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corrected for the radial dilution effect and the Johnston-Ogston effect by the procedure described by DE LALLA AND GOFMAN[12]. Base lines of the sedimentation patterns were obtained by ultracentrifugation of the buffers under the same conditions as the protein solutions.

RESULTS AND DISCUSSION

The results of these experiments are presented in Fig. 1 and Table I. For both serum and the γ-globulin fraction four peaks (7, 12, 19, and 28 S) were evident in the barbital buffer (excluding the slow albumin peak in the serum). The pattern was remarkably different in the acetate pH 4.25 buffer since only two globulin peaks were present: a small 19 S peak, such as found in normal serum, and the large symmetrical, relatively sharp 7 S globulin peak. This difference represents complete, or almost complete, dissociation of the macroglobulin components in the acid buffer. The reversibility of the dissociation is indicated by the typical pattern in experiment E, in which the protein was initially dissolved in and dialysed against the acid buffer before final and exhaustive dialysis against barbital buffer. Calculations, however, indicate that there is a quantitative difference between the parent species and the pH reconstituted species; the reconstituted species has a smaller proportion of the 12 S component, and the component which should sediment at 19 S sediments at 15.2 S. Whether this implies a lack of complete reversibility is not certain. The presence of the small amount of a 19 S component in acid buffer may indicate that the normal serum 19 S component is precipitated with the γ-globulin, and is a single protein entity and not an aggregate of 7 S γ-globulin, which the major portion of the macroglobulin seemed to be.

Further physical and chemical studies on this protein are being performed here.

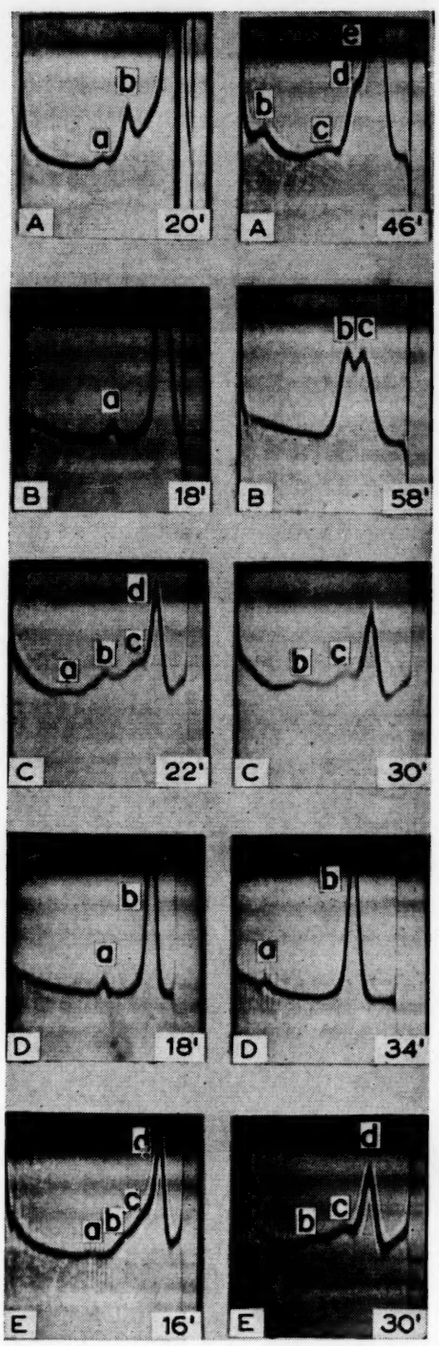

Fig. 1. Ultracentrifuge sedimentation patterns for the experiments described in Table I and the text. The letters designating the experiments and the times of sedimentation (in minutes) at 59,780 rev./min are given in the lower left and lower right corners of each plate, respectively. The components are sedimenting from right to left and the peaks are labeled "a", "b", "c", etc., in order of their appearance.

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ACKNOWLEDGEMENT

For the opportunity of studying this serum, we are grateful to Drs. D. L. HOWIE and E. ADELSON, who are undertaking hematological studies on this patient at the Walter Reed Army Institute of Research.

SUMMARY

The serum and γ-globulin from a patient with macroglobulinemia were examined with the ultracentrifuge. High concentrations of the 12 S, 19 S, and 28 S components were noted in solutions of barbital buffer at pH 8.6. Dissociation of the bulk of these heavy components to a 7 S component occurred in acetate buffer of pH 4.2. Replacement of the acetate buffer by barbital buffer resulted in the reappearance of the heavy components. These findings are consistent with the concept that abnormal macroglobulins are aggregates of a 7 S γ-globulin. The portion of the 19 S component which did not dissociate in acid buffer may represent the γ-globulin present in normal serum.

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PHÉNOLSTÉROIDES URINAIRES

I. MÉTHODE DE DOSAGE

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Sous le terme générique de phénolstéroïdes urinaires ou de folliculine, nous désignons les œstrogènes naturels urinaires dont cinq représentants ont été individualisés dans l'urine humaine (œstriol, œstrone, œstradiol-17β, 16-épiœstriol, 16α-hydroxy-œstrone); ils donnent la réaction de KOBER.

La quasi-totalité de ces œstrogènes est conjuguée; les conjugués sont principalement des glycuronides, une petite fraction étant sous forme d'ester sulfate[1, 2]. La glycuronidase et l'arylsulfatase, qui ont été mises en évidence dans le suc digestif d'*Helix pomatia* par HENRY *et coll.*[3], libèrent la totalité des phénolstéroïdes urinaires.

KOBER[4] en 1931 a décrit une réaction colorée très sensible et relativement spécifique des œstrogènes. De nombreux auteurs ont utilisé des modalités variées de cette réaction pour apprécier le taux des phénolstéroïdes urinaires. On trouvera dans nos publications antérieures[5, 6], dans celles de BROWN[7], dans des revues générales[8–10], l'ensemble des recherches sur l'application de la réaction de KOBER à l'évaluation des phénolstéroïdes urinaires. Certains auteurs préfèrent dénommer les substances ainsi mesurées "chromogènes de Kober". Les recherches de BROWN[11], de BAULD[12, 13], de DICZFALUSY[14] apportent la preuve que les chromogènes interférents des extraits phénoliques urinaires méthylés, purifiés par chromatographie sur colonne d'alumine, après hydrolyse chlorhydrique, ont une absorption linéaire entre 470 et 550 mμ. Ces chromogènes interférents peuvent être éliminés par l'équation de correction d'ALLEN[15], dont nous avons proposé l'application, avec CRÉPY en 1950[16], à la mesure photométrique de la coloration de KOBER. En définitive, les chromogènes de KOBER sont constitués d'œstriol, d'œstrone et d'œstradiol-17β.

Lorsque l'on substitue à l'hydrolyse chlorhydrique, l'hydrolyse par une préparation de suc digestif d'*Helix pomatia*, on ne libère, à côté des stéroïdes, qu'une quantité faible de pigments interférents dont la majeure partie est éliminée au cours des lavages. Nous avons vérifié que ces chromogènes présentaient une absorption linéaire entre 470 et 550 mμ et qu'ils pouvaient être éliminés par la simple application de l'équation de correction d'ALLEN, lorsque le taux des œstrogènes urinaires, exprimés en équivalents d'œstrone, était supérieur à 100 μg pour 1000 ml. Au-dessous de 100 μg pour 1000 ml, il est nécessaire de purifier l'extrait en lui appliquant soit la saponification en milieu sodique *N* préconisée par BAULD[13], soit la méthylation selon le procédé de BROWN[11], soit, dans certains cas d'urines particulièrement pigmentées, l'association de ces deux modes de purification. Le taux des chromogènes devient ainsi très faible et une quantité aussi petite que 20 μg pour 1000 ml de phénolstéroïdes peut être évaluée avec une approximation qui est suffisante pour les besoins de la clinique. Au-dessous de 20 μg pour 1000 ml il est nécessaire d'utiliser la chromatographie d'adsorption ou de partage selon les techniques mises au point par BROWN[11]

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et par BAULD[12, 13]. En conséquence, nous estimons que dans le cas des extraits phénoliques des urines humaines purifiés par chromatographie ou obtenus après hydrolyse enzymatique, on est autorisé à exprimer le résultat sous la dénomination de phénolstéroïdes ou de folliculine.

Tous les auteurs qui ont utilisé la méthode de BROWN sont unanimes à reconnaître sa spécificité et son exactitude; elle apporte une solution au problème du dosage chimique des œstrogènes urinaires. Elle a cependant l'inconvénient de comporter un grand nombre de stades de purification et de chromatographie et d'être d'une exécution délicate; de ce fait, elle n'est pas adaptée aux impératifs de la biologie clinique et elle doit être réservée au laboratoire de recherche, comme le souligne MARRIAN[17]. En raison de l'importance du dosage des phénolstéroïdes dans l'exploration des gonades et du placenta, il est nécessaire de disposer d'une méthode plus simple et plus rapide pour la routine clinique. La présente méthode atteint cet objectif; nous publierons ultérieurement les recherches expérimentales qui ont permis sa mise au point au cours de ces quatre dernières années. Dans l'article suivant, nous donnerons les résultats obtenus chez différents groupes de sujets, ainsi que les méthodes que nous avons standardisées en vue de l'exploration des gonades et du placenta. Pour réaliser l'exploration du corps jaune menstruel ou gestatif, il y avait intérêt à coupler les dosages du pregnandiol et des phénolstéroïdes qui représentent les deux activités endocriniennes du corps jaune. A cet effet, une méthode d'hydrolyse et d'extraction de ces deux catégories a été mise au point; le pregnandiol est évalué dans l'extrait neutre par la technique de CRÉPY *et coll.*[18].

MÉTHODES ET RÉSULTATS

Principe du dosage

Les œstrogènes conjugués des urines fraîches sont hydrolysés par la β-glycuronidase et l'arylsulfatase d'*Helix pomatia*. Les extraits éthérés, lavés par une solution de carbonate de sodium, sont séparés en fraction phénolique et en fraction neutre par NaOH *N*.

Après la purification des extraits phénoliques, qui dépend de la concentration de ces substances, on pratique sur l'extrait final la réaction de KOBER selon une modalité nouvelle et on effectue les mesures photométriques à 476, 516 et 556 mμ; après application de l'équation d'ALLEN, on exprime les résultats en μg d'équivalents d'œstrone en se référant à une courbe d'étalonnage*.

A. Urines ayant une concentration en phénolstéroïdes inférieure à 100 µg pour 1000 ml

Matériel

Étuve à 37° pour hydrolyse enzymatique

Colonnes d'extraction. On utilise pour l'extraction des urines, des colonnes verticales mesurant 800 mm de haut et 24 mm de diamètre intérieur (Fig. 1)**. Elles présentent à leur partie inférieure un robinet et sont ouvertes à la partie supérieure de façon à recevoir un entonnoir qui est terminé par un tube semi-capillaire; le dia-

* Nous remercions le Professeur VELLUZ de nous avoir fourni des échantillons d'œstrone et les docteurs R. H. CALLOW et J. B. BROWN de nous avoir procuré des échantillons purifiés des trois œstrogènes méthylés.

** Ce procédé d'extraction a été mis au point par CRÉPY[19] dans notre laboratoire.

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mètre de ce conduit est calculé de façon à assurer en 90 secondes l'écoulement de 100 ml de liquide. Un support circulaire et rotatif permet de placer côte à côte 6 à 12 colonnes. Au laboratoire d'hormonologie, nous avons réalisé avec M. EGOROFF un appareil semi-automatique permettant l'extraction simultanée de 12 échantillons urinaires de 100 ml (Fig. 2); il est fixé au mur d'une chambre convenablement ventilée et refroidie. De l'air comprimé permet le reflux automatique de la phase aqueuse à la partie supérieure de l'appareil, de façon à réaliser plusieurs passages

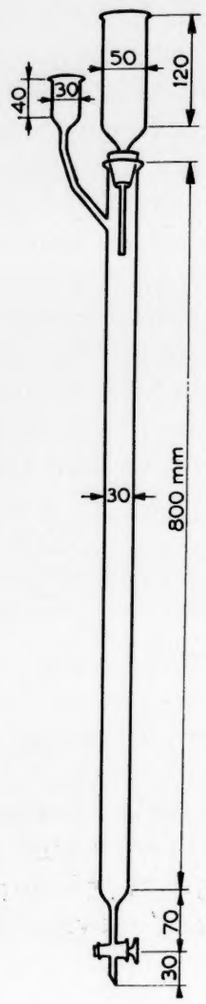

Fig. 1. Colonne d'extraction.

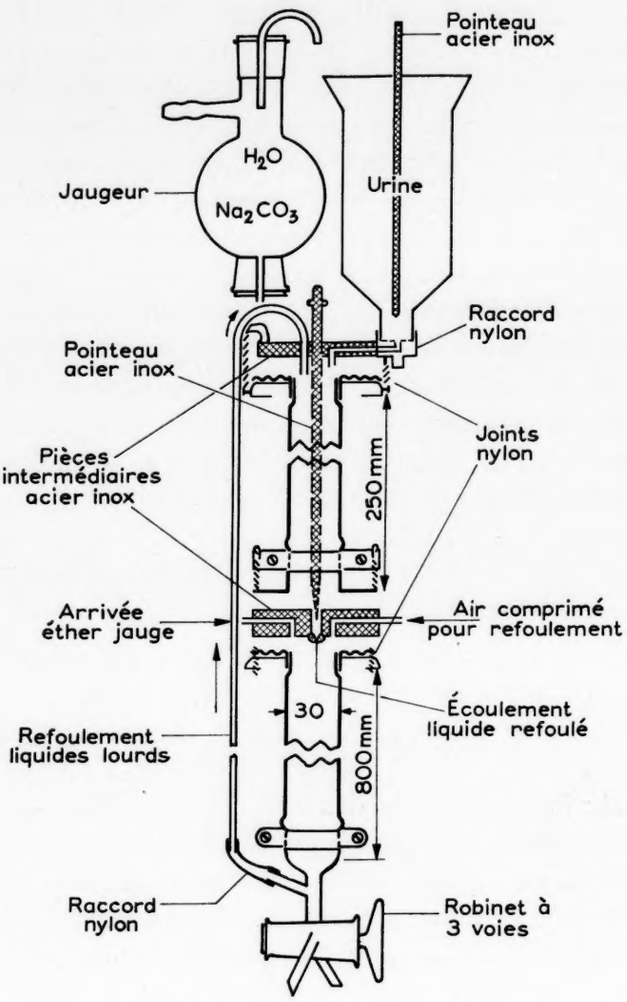

Fig. 2. Croquis schématisant un élément de l'appareil semi-automatique pour l'extraction des phénolstéroïdes.

à travers la phase éthérée. Les tubes semi-capillaires des entonnoirs sont obturés par des pointeaux commandés par un levier. Des réservoirs jaugés de 75 ml situés à la partie supérieure sont remplis automatiquement par les différentes solutions qui peuvent être introduites ensuite dans l'appareil.

Ampoules à décantation de 500 ml.

Bain de sable avec prise de vide et arrivée d'azote pouvant recevoir 12 fioles coniques de 250 ml.

Batterie d'extracteurs "Isomantle" pouvant recevoir les ballons destinés à la saponification et leurs réfrigérants.

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Ballons de saponification piriformes. Volume: 400 ml; diamètre extérieur: 90 mm maximum; rodage sphérique "Quickfit".

Bain-marie à circulation d'eau chaude permettant d'évaporer à sec l'éther de 6 à 12 fioles coniques de 250 ml.

Fioles jaugées de 5 ml pour la reprise des extraits par l'éthanol.

Tubes à hémolyse de 10 mm de diamètre intérieur, 70 mm de hauteur et ayant une paroi d'un mm d'épaisseur.

Bouchons de polyéthylène pour les tubes à hémolyse.

Appareil destiné à évaporer sous azote la solution éthanolique d'hydroquinone et d'extrait urinaire. Il est constitué d'une pomme de douche; à chaque trou est vissé un petit ajutage métallique sur lequel est fixé par l'intermédiaire d'un tuyau de polyvinyle un tube de verre de 2 mm de diamètre intérieur. On place au bain-marie, les tubes à hémolyse contenant la solution éthanolique, après avoir engagé dans chacun d'eux un de ces tubes de verre. L'azote arrive sous pression par la partie supérieure; 24 tubes peuvent être évaporés simultanément au bain-marie bouillant.

Agitateur à secousses. Pour réaliser en série la méthylation des extraits, nous utilisons un agitateur en matière plastique de notre fabrication; celui-ci secoue le liquide des fioles coniques placées verticalement par un va-et-vient à une cadence variable de 1 à 10 secousses par minute et communique au liquide un mouvement de rotation.

Bain-marie réglé à 37° pour la méthylation des extraits.

Spectrophotomètre Unicam SP 600.

Cuves à faces parallèles de 10 × 10 mm de section et 47 mm de hauteur. Une cale spéciale de hauteur variable selon l'appareil permet de soulever les cuves dans le porte-cuves afin que le faisceau lumineux puisse traverser convenablement le liquide.

Solvants et réactifs

Éther éthylique pur pour anesthésie (*Séciven*). On le distille immédiatement avant l'usage; on vérifie qu'il est exempt de peroxydes en ajoutant à 10 ml d'éther, 1 ml d'une solution aqueuse d'anhydride chromique (R.P.) à 1%; après agitation, la couche éthérée ne doit pas se colorer immédiatement en bleu. Si l'éther contient des peroxydes, il est nécessaire de le distiller et de l'utiliser dans la journée*.

Ethanol absolu exempt d'aldéhydes pour la reprise des extraits. Ce solvant est traité selon le procédé préconisé par BAULD [20]. L'éthanol absolu du commerce est porté à l'ébullition à reflux pendant 12 heures en présence de 5% (p/v) de poudre de zinc et de 5 % (p/v) de pastilles de NaOH. L'éthanol est ensuite filtré et additionné de 2.5‰ (p/v) de chlorhydrate de *m*-phénylènediamine. On le laisse reposer 7 jours à l'abri de la lumière, en le remuant une fois par jour; après filtration, on le distille deux fois en éliminant les fractions de tête et de queue. Lorsqu'on ne pratique pas ce traitement, il est nécessaire de vérifier l'éthanol "pur exempt d'aldéhydes".

Tampon acétate, 2 M, pH = 5.2 ± 0.1. Acide acétique glacial (R.P.). Acétate de sodium, pur cristallisé (R.P.).

Carbonate de sodium, pur, anhydre (Billault). Une solution à 9% est préparée à

* Il est essentiel d'utiliser un éther d'excellente qualité afin d'éviter les chromogènes interférents qui ne peuvent être éliminés, même en purifiant un éther de mauvaise qualité.

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chaud; après refroidissement, on l'additionne d'une faible quantité de bicarbonate de sodium suffisante pour atteindre le pH 11.2 ± 0.1.

Bicarbonate de sodium (R.P.).

Soude en pastilles pour analyse. Solution *N*; solution 0.4 *N* (1.6% de BROWN); solution 5 *N* (20% de BROWN).

Diméthylsulfate Merck, distillé 2 fois et utilisé dans les 5 jours qui suivent.

Acide borique cristallisé pour analyses.

Eau oxygénée à 110 volumes (Electro), pure pour analyse (R.P.), conservée à la glacière et portée à la température de la pièce avant emploi.

Hydroquinone purissime, Merck; solution éthanolique à 4% (p/v).

Acide sulfurique concentré, pour analyse (R.P.). Solution à 30% (v/v). Solution *N*/20 et *N*/10 environ. Il n'est pas nécessaire de déterminer le titre de ces solutions avec précision, car elles sont destinées aux lavages.

Acide sulfurique concentré pour analyse (Merck). Solution 26 *N*; solution à 31.5% (v/v) contenant 0.01 g/100 ml de nitrate de sodium (R.P.). Cette solution permet à la fin de la réaction de KOBER de ramener la concentration en H_2SO_4 aux environs de 45% afin de faciliter l'oxydation.

Mode opératoire

(1) Hydrolyse des œstrogènes conjugués urinaires. On part approximativement du dixième du volume urinaire et d'un volume minimum de 100 ml. Lorsque le volume urinaire de 24 h est inférieur à 750 ml et que le taux de la créatinine est normal, on le complète à ce volume.

Les urines sont amenées par addition d'acide acétique à un pH voisin de 5.2 et additionnées de 5% d'une solution tampon acétate 2 *M* de pH 5.2. On ajoute par ml 700 unités de glycuronidate de phénolphtaléïne (PPG) (Fishmann) de β-glycuronidase et environ 300 unités de sulfate de phénolphtaléïne (PPS) (Whitehead) de sulfatase d'une préparation de suc digestif d'*Helix pomatia**. Les urines sont placées à l'étuve pendant 18 à 24 h.

(2) Extraction des urines. 150 ml d'éther éthylique sont introduits, par l'ajutage latéral, dans les colonnes décrites ci-dessus. Si l'on désire pratiquer simultanément l'extraction du pregnandiol, on introduit 150 ml d'un mélange comprenant 4 parties d'éther et une partie d'alcool à 97° exempt d'aldéhydes.

L'hydrolysat urinaire est versé dans l'entonnoir supérieur et il traverse en pluie fine, dans le temps requis, la hauteur de la couche éthérée; lorsqu'il est rassemblé à la partie inférieure de la colonne, on le décante et on l'introduit à nouveau dans l'entonnoir supérieur. Sept passages successifs sont ainsi réalisés, permettant l'extraction de 98% de l'œstriol et de 95% de l'œstrone; la perte étant très faible et sensiblement constante, il n'y a pas lieu en routine clinique de refaire une seconde extraction.

Lorsque le dixième du volume de 24 heures dépasse 130 ml, on le divise en 2 parties que l'on fait passer successivement dans l'extracteur.

Un même manipulateur peut réaliser simultanément l'extraction de 6 à 12 hydrolysats urinaires selon le clavier de colonnes dont il dispose. Après avoir décanté et rejeté l'extrait urinaire, l'éther (ou le mélange éther–alcool) est lavé en versant dans l'entonnoir successivement 2 volumes de 75 ml de la solution de Na_2CO_3; ces solutions

* Nous utilisons des préparations d'*Helix pomatia* fournies par l'Industrie Biologique Française.

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sont décantées et rejetées et on fait ensuite traverser la phase éthérée 3 fois de suite par chaque fois de 50 ml de soude normale. Les 150 ml de solution sodique sont réunis, après décantation soigneuse, dans un ballon de 400 ml.

Si on désire doser le pregnandiol par la méthode de CRÉPY *et coll.*[18], la phase éthéro-alcoolique est lavée par 50 ml de H_2SO_4 $N/30$, puis par 50 ml d'eau. L'éther–alcool est ensuite évaporé à sec.

(3) Saponification de l'extrait phénolique. La solution sodique normale est placée quelques instants dans un bain-marie afin de chasser l'éther qui y est dissous; on porte ensuite à ébullition à reflux pendant 30 min.

Après refroidissement, on extrait la solution sodique dans une ampoule à décantation par 100 ml d'éther que l'on rejette; on neutralise par H_2SO_4 concentré en présence de rouge de phénol jusqu'au virage au jaune et on sature la solution par du bicarbonate de sodium (10 g), afin d'obtenir un pH voisin de 8.2 et une force ionique élevée. On extrait ensuite cette solution alcaline par 200 ml d'éther dans une ampoule à décantation. Cette extraction peut aussi se faire dans les colonnes: on y introduit 200 ml d'éther et on fait passer la solution précédente par portions de 75 ml, 4 fois de suite chacune. La solution éthérée est lavée par 50 ml de H_2SO_4 $N/20$ et 50 ml d'eau distillée (1 seul passage si on utilise les colonnes). On évapore à sec l'éther après avoir ajouté 0.1 ml d'une solution éthanolique d'hydroquinone à 4%. On chasse l'eau résiduelle à 100° et sous vide, afin d'éviter l'oxydation de l'hydroquinone.

Nous avons adopté pour la routine clinique ce mode de purification; nous discuterons plus loin ses avantages et ses inconvénients en le comparant à la méthode de BROWN.

(4) Purification de la solution sodique par méthylation selon Brown. Dans la majorité des cas, en routine clinique, la purification des extraits par saponification est suffisante; cependant, lorsque les urines sont très pigmentées et que la mesure photométrique dépasse une densité optique de 0.8, il est nécessaire de procéder à la méthylation de l'extrait précédent.

La solution sodique saponifiée ou non est neutralisée et saturée par le bicarbonate de sodium, extraite par l'éther et évaporée à sec sans addition préalable d'hydroquinone, selon la technique précédemment décrite. Lorsque l'on pratique successivement la saponification et la méthylation, la solution sodique n'est pas traitée par l'éther après saponification, mais elle est directement neutralisée, tamponnée et extraite par l'éther.

Après évaporation à sec de cet éther dans une fiole conique de 250 ml, on ajoute à l'extrait sec 50 ml de solution sodique 0.4 N et ensuite 0.9 g d'acide borique de façon à tamponner la solution. Le pH, qui est de 12.7 au départ, se maintient en cours de méthylation entre 10 et 11. La fiole conique contenant cette solution est placée sous une hotte munie d'un bon tirage et on y introduit, avec les précautions d'usage*, 1 ml de diméthylsulfate fraîchement distillé en utilisant une pipette à remplissage automatique. On agite énergiquement jusqu'à dissolution, soit à la main, soit de préférence dans un agitateur spécial; on place alors la fiole conique au bain-marie à 37° pendant 30 min. Après ce délai, on ajoute 2 ml de NaOH 5 N et on procède à une seconde méthylation en ajoutant 1 ml de diméthylsulfate; on agite comme précédemment et on place la fiole conique au bain-marie pendant 20 min.

* La manipulation du diméthylsulfate est dangereuse pour la peau et les muqueuses.

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Afin d'oxyder et de détruire les pigments, on ajoute à la solution refroidie 10 ml de soude 5 *N*, puis 2.5 ml de H_2O_2 à 110 volumes; la réaction est instantanée.

La solution sodique provenant de la méthylation est agitée dans une ampoule à décantation avec 2 fois 50 ml d'éther fraîchement distillé; on peut également pratiquer l'extraction dans une colonne verticale avec 120 ml d'éther (7 passages). Les extraits éthérés réunis sont lavés avec 50 ml de H_2SO_4 *N*/20 (1 passage) et 50 ml d'eau distillée (1 passage).

L'extrait éthéré est recueilli dans une fiole conique de 250 ml; on lui ajoute 0.1 ml de solution éthanolique d'hydroquinone à 4%, et on évapore à sec au bain-marie à 45°. On chasse l'eau résiduelle à 100° et sous vide afin d'éviter l'oxydation de l'hydroquinone.

(5) Colorimétrie. L'extrait sec provenant de l'opération (3) ou de l'opération (4) est immédiatement repris plusieurs fois par de l'éthanol absolu dont on amène le volume à 5 ml dans une fiole jaugée. Il importe de ne pas laisser les extraits reposer pour éviter l'oxydation de l'hydroquinone qui se produit rapidement et gêne la colorimétrie.

On introduit dans un tube à hémolyse 2 ml de solution éthanolique en 2 fois et on évapore à sec sous azote dans l'appareil décrit page 279; on ajoute ensuite 0.2 ml de solution éthanolique à 4% d'hydroquinone et on évapore à sec dans les mêmes conditions; ce dosage est fait en double.

A chaque tube à hémolyse contenant l'extrait, on ajoute 0.6 ml d'acide sulfurique 26 *N*. Les tubes sont bouchés par des bouchons de polyéthylène et plongés pendant 20 min dans un bain-marie bouillant; on agite 2 et 5 min après le début du chauffage. Les tubes sont ensuite refroidis dans la glace pilée et on y ajoute 1.4 ml de la solution de $NaNO_3$ dans l'acide sulfurique à 31.5%; la concentration finale optima de 45% est alors atteinte. Les tubes sont replongés dans la glace pendant 30 min et la coloration qui s'est développée est alors stable pendant 3/4 d'heure.

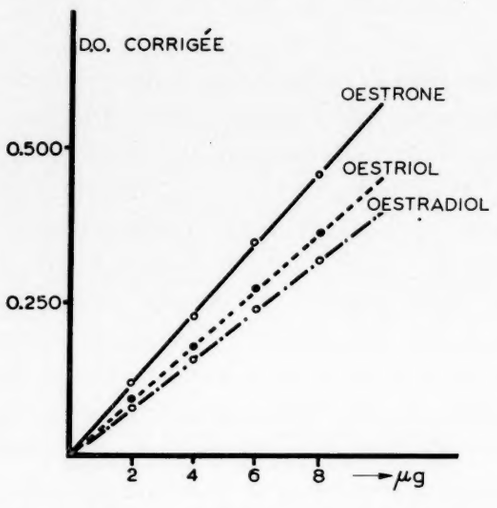

Fig. 3. Droites d'étalonnage de l'œstrone, de l'œstriol et de l'œstradiol obtenues avec notre réactif.

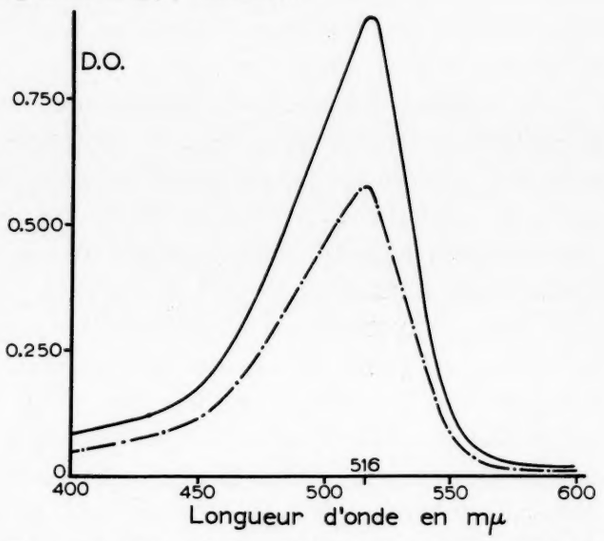

Fig. 4. Courbes d'absorption de 11 μg de 3-méthyl-œstrone (—) et de 9 μg de 3-méthyl-oestriol (—·—).

Les lectures photométriques sont effectuées à 516, 476 et 556 mμ. On applique aux résultats obtenus la formule de correction:

$$DO_c = DO_{516} - \frac{DO_{476} + DO_{556}}{2}$$

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On convertit la densité optique corrigée moyenne en μg de folliculine en se référant à un étalonnage réalisé avec de l'œstrone pure dont on introduit dans 5 tubes en double des quantités croissantes allant de 0 à 8 μg. Les densités optiques corrigées de l'œstrone, de l'œstriol, de l'œstradiol ou de leurs dérivés méthylés à concentration moléculaire égale sont indiqués sur le tracé de la Fig. 3. Les courbes d'absorption de 3-méthyl-œstrone et de 3-méthyl-œstriol sont reproduites sur la Fig. 4. Le photomètre doit être étalonné chaque fois que l'on utilise un nouveau lot d'acide sulfurique Merck.

Les densités optiques corrigées ramenées à 1 μg sont respectivement de 0.058 pour l'œstrone, de 0.045 pour l'œstriol, de 0.040 pour l'œstradiol; le rapport D_c *œstriol*/D_c *œstrone* est voisin de 0.8. La courbe de sensibilité établie selon RINGBOM[21] (Fig. 5) fait apparaître que, au-dessous de 0.2 μg, la sensibilité est nulle et qu'entre 0.2 μg et 10 μg la mesure photométrique se situe dans la partie ascendante de la courbe avec une sensibilité de 0.1 μg.

Discussion

(1) Mode de purification de l'extrait phénolique. Nous avons étudié deux modes de purification de l'extrait phénolique fondés, le premier sur la saponification selon BAULD et le second sur la méthylation selon BROWN, ou éventuellement sur l'association de ces deux procédés.

Pour comparer entre eux ces deux modes de purification, nous avons présenté sur le Tableau I les densités optiques mesurées à 476 mμ correspondant à 40 ml de 6 échantillons urinaires différents; il apparaît que ces deux opérations abaissent la

TABLEAU I

DENSITÉS OPTIQUES MESURÉES À 476 mμ DE SIX ÉCHANTILLONS URINAIRES DIFFÉRENTS, CORRESPONDANT À DIFFÉRENTS MODES DE PURIFICATION

	Densité optique à 476 mμ						
Urine No.	*1*	*2*	*3*	*4*	*5*	*6*	*Moyenne*
Extrait brut	0.700	0.760	0.882	0.792	0.762	1.090	0.831
E. saponifié	0.496	0.460	0.600	0.672	0.488	0.716	0.572
E. méthylé	0.376	0.382	0.600	0.570	0.458	0.650	0.506
E. saponifié puis méthylé	0.340	0.350	0.515	0.420	0.422	0.618	0.441

densité optique à un niveau un peu plus élevé cependant pour la saponification (moyenne D = 0.572), que pour la méthylation (moyenne D = 0.506). La moyenne de la densité optique à 476 mμ des extraits bruts est de 0.831 ce qui permet d'apprécier l'efficacité de ces deux procédés de purification; si on les associe, on obtient une moyenne légèrement inférieure à celle que l'on a avec la méthylation seule (0.441).

Cependant, en analysant les résultats individuels, on se rend compte que cette association peut être intéressante dans le cas de certains échantillons urinaires. Nous reviendrons plus loin sur la récupération des œstrogènes ajoutés à l'urine en fonction du mode de purification. En raison d'une part de la simplicité de la technique de saponification, et d'autre part du gain très faible que l'on obtient en associant les deux procédés, nous avons adopté la saponification dans notre méthode de routine; nous

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ne la complétons par la méthylation que dans les cas où la densité optique, correspondant au 1/25e du volume des urines de 24 h, est supérieure à 0.8, à 476 mμ; cette éventualité ne se produit en principe que dans 2 à 3% des cas. On pourrait améliorer la purification des extraits en associant ces deux procédés et en traitant la solution par l'éther au stade qui se situe après la saponification et avant la méthylation; en effet, dans les essais présentés dans le Tableau I, l'extrait sodique saponifié n'a pas été traité par l'éther comme c'est le cas lorsque l'on pratique la saponification isolément. Nous avons supprimé ce temps opératoire* en raison d'une perte appréciable qu'il entraîne pour l'œstrone, dont 10% environ passent de la solution sodique normale dans la phase éthérée; comme, de son côté, la méthylation entraîne une perte voisine, la récupération de l'œstrone devient alors nettement insuffisante. Cela n'est pas le cas pour l'œstriol, qui ne passe pas de la solution sodique normale dans l'éther; quant à l'œstradiol-17β, sa concentration est relativement négligeable dans les extraits urinaires.

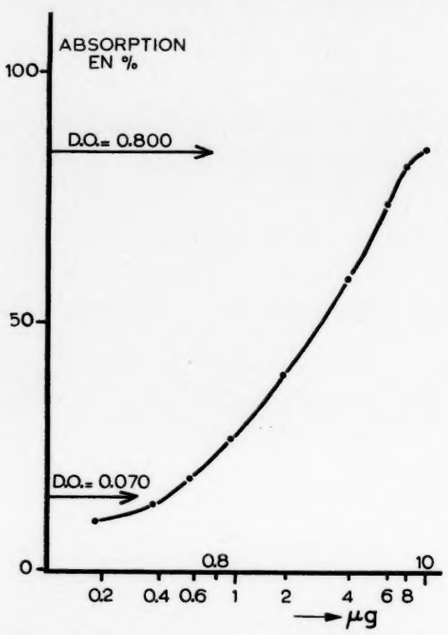

Fig. 5. Courbe de sensibilité établie selon RINGBOM. En ordonnées: absorption = 100 — T%. En abscisses: logarithme de la concentration: μg d'œstrone pour 2 ml de réactif.

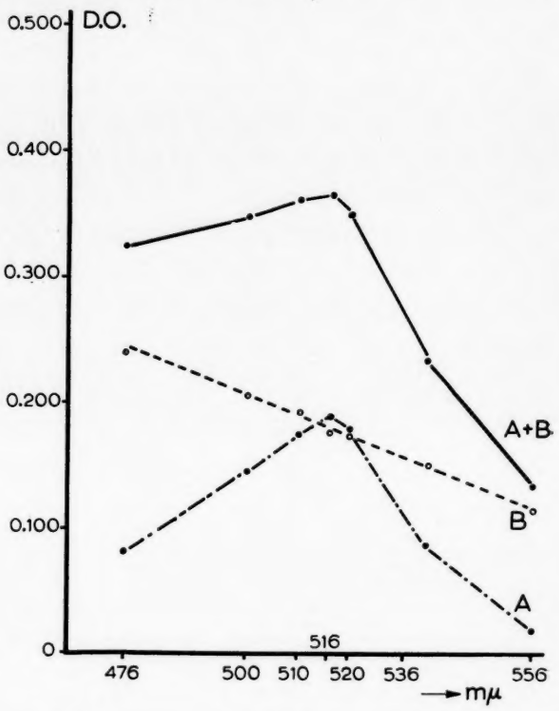

Fig. 6. Décomposition de la courbe d'absorption d'un extrait phénolique.

(2) Décomposition des courbes d'absorption. Pour les 6 échantillons urinaires précédents, dont nous avons donné la densité optique à 476 mμ, nous avons établi une courbe d'absorption entre 476 et 556 mμ. Nous avons calculé dans chaque cas, en équivalents d'œstrone, la quantité de phénolstéroïdes par application de l'équation de correction d'ALLEN, à partir des densités optiques mesurées à 476, 516 et 556 mμ; nous avons retranché, de la courbe d'absorption de l'extrait, la courbe d'absorption de cette quantité d'œstrone calculée. Cela nous a permis de décomposer la courbe d'absorption de l'extrait en deux tracés représentant l'un, la courbe d'absorption des phénolstéroïdes de chaque extrait et l'autre, celle des chromogènes interférents (Fig. 6). Dans

* Nos recherches récentes indiquent que, malgré cet inconvénient, il est préférable d'extraire la solution sodique par l'éther après saponification et avant méthylation; on obtient ainsi des extraits beaucoup plus propres dans le cas d'urines fortement pigmentées.

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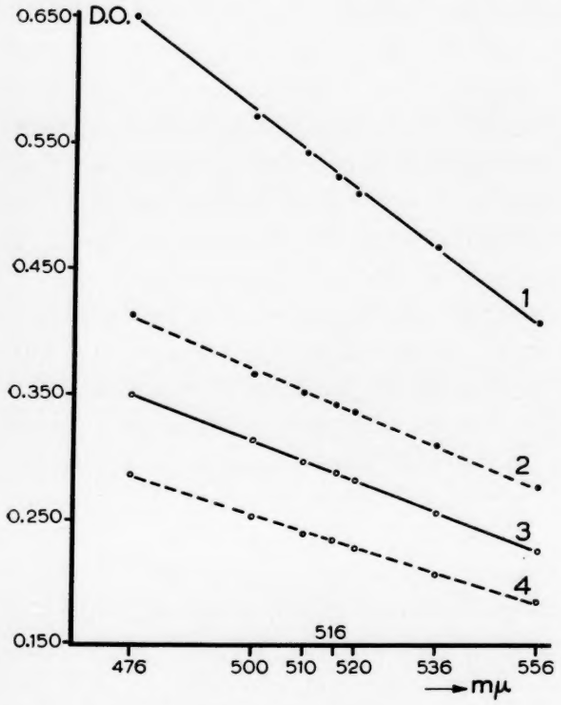

Fig. 7. Courbes d'absorption moyenne des chromogènes interférents. 1. extrait brut, 2. extrait après saponification, 3. extrait après méthylation, 4. extrait après saponification suivie de méthylation.

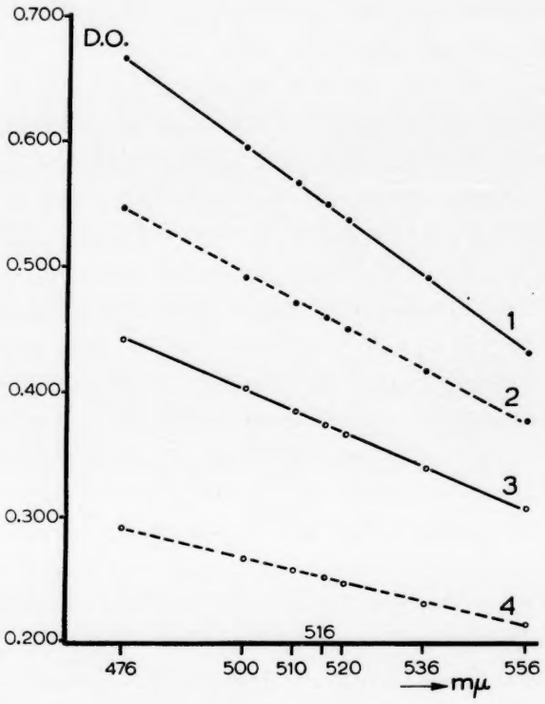

Fig. 8. Courbes d'absorption des chromogènes interférents de l'échantillon urinaire No. 4. Pour la légende *cf.* Fig. 7.

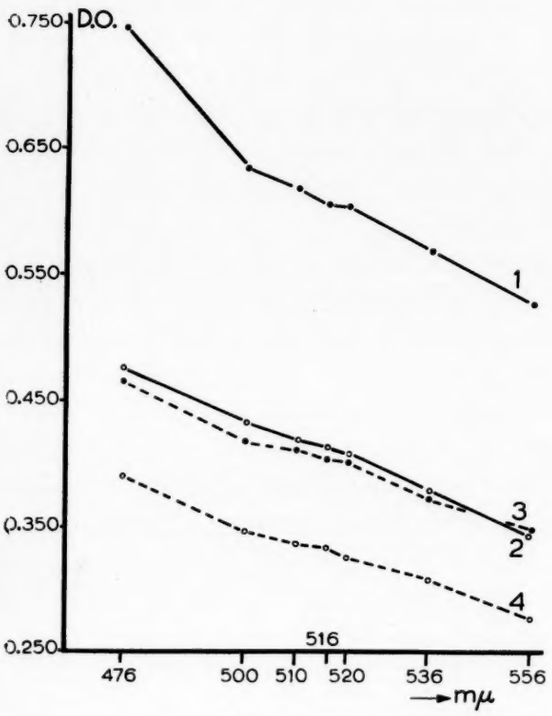

Fig. 9. Courbes d'absorption des chromogènes interférents de l'échantillon urinaire No. 3. Pour la légende *cf.* Fig. 7.

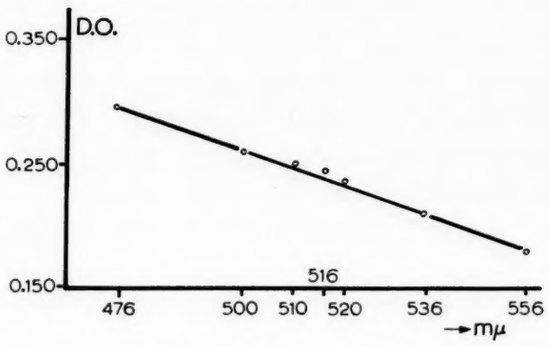

Fig. 10. Courbe d'absorption de l'extrait phénolique d'un mélange d'urines d'enfants impubères après purification par saponification.

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chaque cas nous avons fait cette décomposition pour l'extrait brut, pour l'extrait purifié par méthylation seule, pour l'extrait purifié par saponification seule, et pour l'extrait purifié par les deux opérations associées. La Fig. 7 donne la moyenne des résultats obtenus pour les 6 échantillons urinaires*: il apparaît que les tracés des chromogènes interférents sont sensiblement linéaires après méthylation ou saponification et que le tracé après l'association des deux purifications est parallèle et voisin de celui obtenu après méthylation seule. Le fait qu'individuellement ou collectivement, le tracé des chromogènes soit linéaire nous apporte la preuve que l'équation d'ALLEN peut s'appliquer valablement aux extraits purifiés par saponification ou méthylation. Dans le cas des extraits bruts, 5 fois sur 6 le tracé des chromogènes est sensiblement linéaire, mais son niveau est trop élevé en dehors de la grossesse; lorsque le taux dépasse 100 μg d'équivalents d'œstrone pour 1000 ml, on peut prendre des prises d'essai correspondant à 20 ml par tube, ce qui permet d'éviter les opérations de purification et d'obtenir pour les chromogènes interférents des tracés linéaires à un niveau compatible avec une mesure photométrique valable.

Sur les Figs. 8 et 9, nous avons porté les tracés correspondant aux deux extraits urinaires qui, sur les six étudiés, nous ont donné l'un le meilleur résultat, l'autre le résultat le plus défavorable*. Dans ce dernier cas, c'est surtout le tracé de l'extrait brut qui s'éloigne le plus de la linéarité.

Sur la Fig. 10 nous avons porté la courbe d'absorption de l'extrait phénolique d'un mélange d'urines d'enfants impubères après purification par saponification. Le taux des œstrogènes après application de l'équation de correction d'ALLEN correspond à 0.5 μg. Le tracé est voisin de la linéarité entre 476 et 556 mμ, ce qui apporte un argument supplémentaire en faveur de l'absorption linéaire des chromogènes interférents dans les extraits obtenus après hydrolyse enzymatique.

(3) Exactitude de la méthode. Pour apprécier l'exactitude de la méthode, nous avons ajouté à différents échantillons d'urines d'enfants préalablement hydrolysées par la β-glycuronidase et la sulfatase d'*Helix pomatia* des quantités variables d'œstrone et d'œstriol. Les urines ont ensuite été traités par la méthode que nous avons décrite plus haut, comportant la saponification des extraits sodiques, suivie d'extraction par un volume de 100 ml d'éther. Les résultats sont groupés sur le Tableau II: il apparaît que la récupération de l'œstriol ajouté, entre 35 et 100 μg, est en moyenne égale à 82%. Toutes les opérations ont été faites en double et les pourcentages de récupération varient entre 77 et 86%; pour les 30 expériences réalisées avec 100 μg, l'écart-type est de 3%. Au-dessous de 30 μg, le pourcentage de récupération est plus faible et il devient insignifiant au-dessous de 15 μg, ce qui met en évidence les limites de la méthode. Les résultats de la récupération sont assez symétriques pour l'œstrone, à cette différence près que la récupération n'est plus que de 72% en moyenne. Le faible écart-type, qui est égal à 3% comme précédemment dans le cas des 32 essais pratiqués en double après l'addition de 100 μg d'œstrone, montre l'exactitude de cette méthode.

La sensibilité de cette méthode est améliorée lorsque l'œstrone est ajoutée non pas à l'urine hydrolysée par les enzymes, mais à de l'eau. La récupération est alors de 50% pour 5 μg d'œstrone; elle ne dépasse pas comme dans le cas précédent 72% au-delà de 5 μg (Tableau III).

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* Nous n'avons pas reproduit les six figures par suite du manque de place.

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TABLEAU II

RÉCUPÉRATION AVEC LA MÉTHODE UTILISANT LA SAPONIFICATION

Œstrone									
Quantité ajoutée en μg/1000 ml	5	10	15	20	30	35	50	70	100
Nombre d'expériences	6	6	6	6	2	3	2	3	32
Quantité récupérée en moyenne, μg/1000 ml	0	traces	6.75	12	21.5	25.2	36.5	51	73
Pourcentage	0	traces	45	60	71	72	73	73	73
σ									3
Limites des pourcentages			30–53	55–67	71–71	71–72	73–73	71–73	67–79
Œstriol									
Quantité ajoutée en μg/1000 ml	5	10	15	20	25	35	50	100	
Nombre d'expériences	4	4	4	4	4	4	2	30	
Quantité récupérée en moyenne, μg/1000 ml	0	traces	8.55	13	17.5	28	41	83	
Pourcentages	0	traces	57	65	70	81	82	83	
σ								3	
Limites des pourcentages			56–57	65–65	68–69	80–82	82–82	77–86	

TABLEAU III

RÉCUPÉRATION À PARTIR DE L'EAU AVEC LA MÉTHODE UTILISANT LA SAPONIFICATION

Œstrone					
Quantité ajoutée en μg/1000 ml	5	10	15	20	50
Nombre d'expériences	2	2	2	2	2
Quantité récupérée en moyenne, μg/1000 ml	2.5	5	7.5	10	36
Pourcentage	50	50	50	50	72

TABLEAU IV

RÉCUPÉRATION AVEC LA MÉTHODE UTILISANT LA MÉTHYLATION

	Œstrone					*Œstriol*
Quantité ajoutée en μg/1000 ml	30	35	50	60	70	35
Nombre d'expériences	2	4	1	2	1	4
Quantité récupérée en moyenne, μg/1000 ml	21	25.2	35.5	42	50.5	28.7
Pourcentage	70	72	71	70	72	82
Limites des pourcentages	70–70	69–73		71–69		81–83

TABLEAU V

RÉCUPÉRATION AVEC LA MÉTHODE N'UTILISANT NI SAPONIFICATION NI MÉTHYLATION

Œstrone					
Quantité ajoutée en μg/1000	50	100	200	500	1.000
Nombre d'expériences	2	29	12	6	2
Quantité récupérée en moyenne, μg/1000 ml	41	82	166	410	830
Pourcentage	82	82	83	82	83
σ		3			
Limites des pourcentages	80–83	76–88	74–90	79–86	83–83
Œstriol					
Quantité ajoutée en μg/1000 ml	50	100	200	500	1.000
Nombre d'expériences	2	32	4	4	2
Quantité récupérée en moyenne, μg/1000 ml	40	83	162	420	840
Pourcentage	80	83	81	84	84
σ		2.7			
Limites des pourcentages	80–80	80–84	80–82	82–84	84–84

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Le Tableau IV groupe un plus petit nombre d'expériences au cours desquelles la saponification des extraits a été remplacée par la méthylation. On voit que la récupération de l'œstriol et de l'œstrone est la même que précédemment. Le Tableau V concerne les essais réalisés par la même méthode sans purification. La moyenne de la récupération de l'œstriol est identique à celle des expériences précédentes; en revanche, la moyenne de récupération de l'œstrone passe de 72 à plus de 80%. Autrement dit, la purification par méthylation et saponification n'entraîne pas de pertes appréciables pour l'œstriol, mais une perte de 10% pour l'œstrone.

Nous avons recherché à quelles étapes la perte de l'œstrone ou de l'œstriol se produisait. Dans la méthode no. 2 qui ne comporte ni saponification, ni méthylation, la perte la plus importante se produit au cours du passage de l'œstrone, de l'éther dans la soude normale; l'œstriol au contraire passe quantitativement à ce stade, mais les pertes se produisent essentiellement au cours des lavages. Il serait possible d'améliorer sensiblement la récupération de ces deux œstrogènes en procédant à une double extraction éthérée, tant des urines que de l'extrait sodique neutralisé. En fait, cette opération supplémentaire n'améliore la récupération que de 4% en moyenne; à notre avis, cela ne justifie pas le travail supplémentaire qu'elle entraîne, d'autant plus que la perte qu'elle évite est constante.

Nous n'avons pas étudié la récupération de l'œstradiol-17β en raison des quantités faibles de cet œstrogène dans les urines humaines. En conclusion, la récupération des phénolstéroïdes urinaires est de l'ordre de 75% en moyenne pour des quantités comprises entre 30 et 1000 μg pour 1000 ml. La perte de 25% est très constante et l'erreur appréciée par l'écart-type est de $\pm$ 3 %. Il en résulte que la méthode remplit les conditions que, selon MARRIAN[22], l'on doit exiger d'une méthode de dosage des stéroïdes.

(4) Précision de la méthode. Sur 134 dosages, pratiqués en double par la méthode ne comportant ni saponification, ni méthylation, l'écart-type des variations à été de 2%. Il est égal à 2.5% lorsque les extraits phénoliques sont purifiés par saponification. L'écart-type s'élève à 4.5% lorsque les extraits sont purifiés par méthylation. Ces expériences ont été faites par deux techniciennes bien entraînées. Dans la routine clinique, nous avons constaté que le risque d'erreur individuel était plus important pour la méthylation que pour la saponification; aussi avons-nous choisi la purification par saponification pour la méthode utilisée en clinique.

(5) Spécificité de la méthode. Pour apprécier la spécificité de notre méthode, nous avons pratiqué sur un certain nombre d'échantillons urinaires le dosage de l'œstrone, de l'œstriol et de l'œstradiol-17β par la méthode chromatographique de BROWN et par notre technique comportant la purification par saponification.

On trouvera sur la première colonne des Tableaux VI, VII et VIII le taux des phénolstéroïdes exprimés en μg d'équivalents d'œstrone par 24 h et sur la troisième colonne la somme de l'œstriol, de l'œstrone et de l'œstradiol-17β évalués par la méthode chromatographique de BROWN.

La comparaison des deux catégories de résultats appelle les remarques suivantes: une critique que l'on peut faire à notre procédé provient de ce que le pouvoir chromogène de KOBER de ces trois œstrogènes n'est pas identique; avec notre modalité de coloration, le rapport D_c œstriol/D_c œstrone est voisin de 0.8 et le rapport D_c œstradiol/D_c œstrone de 0.7. Il en résulte que, lorsque l'on traduit les résultats globaux en équivalents d'œstrone, on obtient des valeurs inférieures aux valeurs réelles que donne

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la méthode de BROWN. Il faut également tenir compte du fait que le pourcentage de récupération de l'œstrone est environ de 10% inférieur à celui de la méthode de BROWN. À partir de ces données et des pourcentages moyens de l'œstriol, de l'œstrone et de l'œstradiol que l'on trouve dans les urines, il est possible de calculer un coefficient de correction moyen, qui est de 1.2. Sur ces mêmes tableaux, nous avons donc indiqué dans la deuxième colonne le résultat trouvé en multipliant par ce coefficient le nombre trouvé dans la première colonne.

Afin de faire une comparaison encore plus stricte entre les résultats de notre méthode et ceux de la technique de BROWN, nous avons multiplié par 0.9 le taux de l'œstrone et ceux de l'œstriol et de l'œstradiol respectivement par 0.8 et 0.7 (rapports des D_c indiqués plus haut). En faisant la somme de ces trois valeurs corrigées, on obtient les nombres de la colonne IV, qui doivent théoriquement être égaux à ceux de la colonne I.

TABLEAU VI

COMPARAISON DE LA MÉTHODE PROPOSÉE ET DE LA MÉTHODE DE BROWN AU COURS D'UN CYCLE MENSTRUEL

	Méthode proposée		*Méthode de Brown*				
	I	*II*	*III*	*IV**			
Jours	*Résultat du dosage en µg/24h*	*Taux corrigé*	*Somme des 3 fractions*	*Somme corrigée*	*Œstriol*	*Œstrone*	*Œstradiol*
	A	*A* × 1.2	*B*	0.8 × *C* + 0.9 × *D* + 0.7 × *E*	*C*	*D*	*E*
5e	15	18	8	6.5	4	2.5	1.7
6e	10	12	8.5	7	4	3	1.6
7e	13	15	11	10	4.8	4	1.8
8e	12	14	14	11.5	6.2	5.7	2.5
9e	15	18	21	18	10	9	2.8
10e	19	23	31	25.5	15.4	11.7	4
11e	25	30	39	32	17	15	7
12e	35	42	45	37	19	17	9
13e	48	58	60	49	30	20	10
14e	43	51	65	52.5	31	21	12.4
15e	34	41	—	—	30	—	—
Décalage thermique							
16e	29	35	34	28	19.5	10.7	4
17e	23	28	28	23.5	14	11	3.8
18e	16	19	26	22	13	10	3.5
19e	30	36	37	30.5	17.5	14	5.5
20e	26	31	37	30	17.5	13	6.5
21e	24	29	37	31	16.7	17	3.5
22e	20	24	25	20	13.2	8.4	3
23e	20	24	20	16.5	10	7.4	2.7
24e	16	19	16	13	7	6	2.6
25e	13	16	11	8.5	4.5	3.8	2.4
26e	20	24	10	8	5	3.3	1.6
27e	15	18	9	7	3.8	2.6	2.2

* Les coefficients de correction des trois fractions de BROWN sont calculés en tenant compte, d'une part de la perte supplémentaire de 10% d'œstrone dans notre méthode et d'autre part des pouvoirs chromogènes respectifs des trois œstrogènes avec la modalité de la réaction de KOBER que nous utilisons.

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Le Tableau VI concerne le dosage des œstrogènes fait au cours du cycle menstruel chez une jeune femme normale de 35 ans; il apparaît que lorsque le taux des œstrogènes est égal ou supérieur à 20 μg, il existe une bonne concordance entre les résultats des deux méthodes; en revanche, au-dessous de cette valeur, le tracé s'abaisse beaucoup plus par la méthode de BROWN que par la nôtre.

TABLEAU VII

COMPARAISON DE LA MÉTHODE PROPOSÉE ET DE LA MÉTHODE DE BROWN CHEZ DIFFÉRENTS SUJETS NORMAUX

	Méthode proposée		*Méthode de Brown*				
	I	*II*	*III*	*IV*			
Age (ans)	*Résultat du dosage en μg/24h*	*Taux corrigé*	*Somme des 3 fractions*	*Somme corrigé*	*Œstriol*	*Œstrone*	*Œstradiol*
	A	*A* × *1.2*	*B*	*0.8* × *C* + *0.9* × *D* + *0.7* × *E*	*C*	*D*	*E*
			Filles				
11	10	12	9	7.5	3	4	2
12	5	6	6	4.5	2.5	1	2.5
13	10	12	15	12	9.5	3	2.5
14	15	18	21	17	17.5	3	0.5
14	15	18	14	11	9	2	3
17	13	16	12	9.5	5	4	3
17	12	14	9	7	4.3	2.6	2.1
17	13	16	10	8	5	3	2
Moyenne	12	14	12	9			
			Femmes menopausées				
	15	18	15	12	12	2	1
	15	18	16	13	10	4	2
	10	12	11	9	8	2	1
	10	12	5	4	2	2	1
	9	11	19	14.5	7.5	3	8.5
Moyenne	12	14	13	10.5			
			Hommes				
30	16	19	14	11.5	3.7	7.3	3
26	11	13	17	14	4.3	9.7	3
40	10	12	12	10	4.5	4.8	2.7
34	14	17	10	8	4.5	3.6	1.9
43	20	24	14	11.5	5.8	5.1	3.1
29	15	18	6	5	0.5	3.5	2
33	15	18	6	5	0.4	3	2.6
Moyenne	14	17	11	9			

La concordance des résultats inférieurs à 20 μg est meilleure chez les enfants (Tableau VII); cela indique que le taux des chromogènes interférents est plus faible avant qu'après la puberté. Le Tableau VIII groupe des résultats dépassant 25 μg par 24 h chez différents sujets, l'hyperfolliculinie pouvant être déterminée par une affec-

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TABLEAU VIII

COMPARAISON DE LA MÉTHODE PROPOSÉE ET DE LA MÉTHODE DE BROWN AU COURS DE DIFFÉRENTS CAS D'HYPERFOLLICULINIE

	Méthode proposée		*Méthode de Brown*				
Maladies	*I* *Résultat du dosage en µg/24 h*	*II* *Taux corrigé*	*III* *Somme des 3 fractions*	*IV* *Somme corrigée*	*Œstriol*	*Œstrone*	*Œstradiol*
	A	*A* × 1.2	*B*	0.8 × *C* + 0.9 × *D* + 0.7 × *E*	*C*	*D*	*E*
			Cas divers				
Femme 69 ans dermatomyosite	25	30	27	21.5	13	6	8
Atrophie testiculaire	30	36	21	17	13	3.5	4.5
Hystérectomie avec conservation de l'ovaire droit	40	48	60	48	55	3	2
Arrhénoblastome	40	48	45	37	17	22	6
Maladie de CUSHING	50	60	55	46	20	27	8
	Hommes après stimulation par gonadotropines chorioniques						
Moyenne							
2e et 3e jours	32	38	49	41	13.5	27	8.5
4e et 5e jours	35	42	42	34.5	12	20	10
6e et 7e jours	25	31	37	30	16	12.5	8.5
Moyenne générale	34	41	42	35			

tion endocrinienne, par l'administration de gonadotropines chorioniques, ou par un début de grossesse. On constate qu'il existe une bonne correspondance dans l'ensemble entre les résultats des colonnes I, IV d'une part et II, III d'autre part; il existe cependant parfois des différences sensibles, mais elles n'affectent pas, d'une façon appréciable, la valeur séméïologique du résultat.

On peut donc conclure de l'ensemble de ces données que la méthode que nous proposons présente une bonne spécificité, surtout si les taux de folliculine dépassent 30 µg pour 1000 ml. L'erreur due aux chromogènes interférents, faible chez l'enfant, demeure en règle générale inférieure à 10 µg dans le cas des urines contenant un taux faible de folliculine. On peut pallier le déficit qui est dû aux pouvoirs chromogènes différents des trois œstrogènes en multipliant par 1.2 le résultat exprimé en équivalents d'œstrone. Même s'il existe dans certains cas des différences sensibles entre les résultats obtenus par la méthode de BROWN et par notre méthode, lorsque les taux de folliculine sont compris entre 30 et 100 µg, elles n'affectent pas la valeur séméïologique des résultats.

B. Urines de grossesse

(1) Premier trimestre de la grossesse

Dès le début de la grossesse, le taux des phénolstéroïdes atteint et dépasse rapidement 100 µg par 24 h; aussi, pour des raisons que nous avons exposées précédemment, la proportion des chromogènes interférents par rapport aux chromogènes de KOBER étant plus faible, la purification par saponification de l'extrait est inutile.

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On partira de 100 ml d'urine et on procèdera comme précédemment, à la seule différence près que la solution sodique normale sera réextraite directement après avoir ramené son pH à 8.2 par addition de $NaHCO_3$. Pour la mesure colorimétrique, on utilisera une prise d'essai contenant de 1 à 5 μg d'équivalents d'œstrone, en se basant sur les valeurs moyennes de l'excrétion des phénolstéroïdes entre la cinquième et la quinzième semaines après les dernières règles.

(2) Deuxième et troisième trimestres de la grossesse

Au terme du premier trimestre de la grossesse, les quantités de phénolstéroïdes s'élèvent au-dessus de 1000 μg par nycthémère; aussi est-il possible en pratique de faire le dosage sur un échantillon de 10 ml d'urine.

a. Hydrolyse. L'hydrolyse enzymatique est réalisée par 2000 unités PPG de glycuronidase et 1000 unités PPS de sulfatase par ml d'urine, à pH 5.2 (voir plus haut pour tamponner). Au-delà de la 20e semaine de la grossesse (à dater du premier jour des dernière règles), les urines sont diluées de façon à amener la concentration des phénolstéroïdes à moins de 8000 μg pour 1000 ml car, au-delà de celle-ci, l'hydrolyse enzymatique n'est plus quantitative en 18 h.

b. Extraction. Elle est réalisée selon le même principe que précédemmment dans des colonnes de petit calibre mesurant 425 mm de haut et 13 mm de diamètre intérieur. Le diamètre du capillaire de l'entonnoir est calculé de façon à assurer en 17 secondes l'écoulement de 10 ml de liquide; 12 colonnes sont disposés autour d'un support circulaire rotatif et muni d'un compte-tours (cet appareil a été réalisé par CRÉPY *et coll.*[23]).

On introduit dans chaque colonne 20 ml d'éther exempt de peroxydes; on verse dans l'entonnoir supérieur l'hydrolysat urinaire contenu dans une fiole conique que l'on place au-dessous de la colonne correspondante et on passe à l'extraction suivante. Lorsque le tourniquet a réalisé un tour complet, on revient à la première colonne, on décante l'urine et on la reverse dans l'entonnoir supérieur et ainsi de suite. On effectue huit passages successifs, soit huit tours de tourniquet, ce qui est suffisant pour obtenir l'équilibre de partage et 95% des phénolstéroïdes dans la phase éthérée. Les urines sont alors soigneusement décantées et rejetées.

On fait ensuite passer en une seule fois, dans chaque colonne, 15 ml d'une solution de Na_2CO_3 ajustée à pH 11.2; ces eaux de lavage sont décantées et rejetées.

L'extraction des phénolstéroïdes est réalisée par 10 ml de NaOH *N*, en deux portions de 5 ml que l'on fait passer chacune trois fois à travers la phase éthérée. On mélange les solutions sodiques et on lave une dernière fois l'éther par 1 ml de NaOH *N* qu'on ajoute à la solution précédente.

On neutralise la soude par H_2SO_4 à 30% selon la même modalité que précédemment; on ajoute environ 5% (p/v) de $NaHCO_3$ afin d'amener le pH aux environs de 8.2.

On introduit dans les colonnes d'extraction 20 ml d'éther fraîchement distillé et on procède, comme précédemment, à l'extraction éthérée (8 passages). L'éther est lavé par 5 ml de H_2SO_4 *N*/20 (un seul passage) et par une fois 5 ml d'eau distillée (un seul passage). L'éther est ensuite évaporé à sec; la reprise de l'extrait dans 5 ml d'éthanol et la colorimétrie sont effectuées comme ci-dessus en faisant une prise d'essai convenable.

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DISCUSSION ET CONCLUSIONS GÉNÉRALES

Du point de vue analytique, le problème du dosage de l'œstrone, de l'œstradiol et de l'œstriol est résolu par la méthode de BROWN et par celle de BAULD, dont la spécificité et l'exactitude, la précision et la sensibilité permettent d'évaluer des quantités totales d'œstrogènes de l'ordre de 5 μg/l. Ces méthodes, en dépit des performances indiquées par les auteurs, sont d'une exécution longue et très délicate, et elles n'apportent pas une solution au problème du dosage des œstrogènes en routine clinique. La question que nous nous sommes posée et en raison de laquelle nous avons réalisé ce travail était de savoir s'il était possible de mettre au point une méthode de dosage des œstrogènes urinaires sans procéder à une purification chromatographique et si les qualités d'une telle méthode pouvaient être considérées comme suffisantes pour son application à la clinique. En remplaçant l'hydrolyse acide par l'hydrolyse par les enzymes du suc digestif d'*Helix pomatia* (β-glycuronidase et sulfatase) on obtient des extraits éthérés beaucoup plus propres et il devient possible, dans ces conditions, de simplifier considérablement la purification des extraits.

Certains auteurs[24,25], en utilisant les méthodes chromatographiques, ont trouvé que les enzymes de la patelle contaminent les extraits par des impuretés qui gênent le dosage de la fraction œstriol. Cet inconvénient existe exceptionnellement avec certains lots de suc digestif d'*Helix pomatia* lorsque l'on utilise la méthode chromatographique*. C'est pourquoi ces lots doivent être éliminés ou purifiés. Lorsque l'on prend ces précautions, l'addition aux urines de suc d'*Helix pomatia* n'introduit pas de chromogènes gênants. En outre, les exigences de notre méthode sont moins grandes que celles de la méthode de BROWN, car nous utilisons une portion aliquote beaucoup plus faible (40 ml au lieu de 200 ml pour un volume général de 1200 ml).

Dans un travail sur l'hydrolyse enzymatique des œstrogènes conjugués qui est en cours de rédaction, nous avons constaté que le glucose et certains médicaments diminuent le taux de la folliculine lorsqu'on pratique une hydrolyse chlorhydrique, alors que cet inconvénient n'existe pas avec l'hydrolyse enzymatique. Les œstrogènes sont en majeure partie sous forme de glycuro-conjugués et, à la fin de la gestation, le taux des sulfo-conjugués n'excède pas 10 pour 100; il est cependant possible que le pourcentage des sulfo-conjugués ne soit pas le même en dehors de la grossesse ou au cours de celle-ci, qu'il soit variable chez les différents sujets et que le rôle de la phénolsulfatase soit de ce fait plus important.

Nous avons vu au cours des chapitres précédents que l'exactitude de notre méthode, comme sa précision, évaluées la première à partir des données de la récupération des œstrogènes ajoutés, la deuxième à partir des déterminations faites en double, étaient très satisfaisantes; la spécificité est nettement insuffisante au-dessous de 20 μg pour 1000 ml en équivalents d'œstrone; elle devient satisfaisante au-dessus de 20 μg et excellente à partir de 50 μg. L'erreur, qui est dûe aux pigments interférents, n'excède qu'exceptionnellement 10 μg pour 1000 ml. Il en résulte que les méthodes chromatographiques devront être utilisées pour doser de très faibles quantités. En clinique, l'intérêt de cette recherche est, sinon nulle, du moins très secondaire, et ce qui importe principalement en pratique c'est la recherche de l'hyperfolliculinie. Sa détec-

* Nous n'avons jamais retrouvé cet inconvénient lorsque les fractions d'œstrogènes sont préalablement saponifiées selon la méthode de BROWN[25].

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tion spécifique est réalisable par notre méthode, comme cela est prouvé non seulement par l'étude individuelle des cycles menstruels normaux, mais aussi par de nombreux résultats pathologiques ou encore par la stimulation des gonades des deux sexes par les hormones gonadotropiques chorioniques (HGC). Dans tous ces cas d'hyperfolliculinie, la concordance des résultats obtenus avec la somme des trois fractions du dosage chromatographique de BROWN est satisfaisante.

La récupération des œstrogènes ajoutés est meilleure pour l'œstriol que pour l'œstrone, pour les raisons que nous avons indiquées précédemment; cela est compensé dans une certaine mesure par le fait que le pouvoir chromogène de l'œstrone est plus élevé que celui de l'œstriol.

L'intérêt que présente en clinique le fractionnement des trois œstrogènes principaux n'est pas encore nettement établi; l'administration d'œstradiol-17β à des sujets castrés conduit à l'excrétion des trois œstrogènes dans des rapports qui se rapprochent de ceux que l'on constate au cours des deux phases du cycle menstruel, (BROWN [26]) et il n'existe encore aucun argument solide concernant la valeur séméïologique des rapports entre les trois œstrogènes urinaires; aussi estimons-nous que la méthode de dosage des phénolstéroïdes totaux répond bien aux besoins de la clinique. Une technicienne entraînée, disposant du matériel requis, peut en effet pratiquer une douzaine de dosages quotidiennement.

Si on se réfère à l'opinion émise par MARRIAN [22], une méthode de dosage des stéroïdes peut être considérée comme valable lorsque le pourcentage de récupération des stéroïdes ajoutés est de l'ordre de 75% ou davantage, avec une déviation standard n'excédant pas 10%; notre méthode répond à ces exigences.

Nous avons étudié deux modalités différentes pour le dosage des phénolstéroïdes totaux: la première comportant la saponification selon BAULD [12, 13], la seconde comportant la méthylation de l'extrait selon BROWN [11]. L'expérience prouve qu'en routine clinique la saponification comporte moins de risques d'erreur de la part des techniciennes que la méthylation et qu'elle réalise une purification suffisante dans la quasi-totalité des cas; c'est pourquoi nous l'avons adoptée. Il existe cependant certaines urines pour lesquelles le taux des chromogènes interférents est particulièrement élevé, ce qui justifie une purification supplémentaire par méthylation.

Il résulte de notre expérience personnelle que la méthode de dosage des phénolstéroïdes totaux que nous proposons en routine clinique est suffisamment précise, exacte et spécifique pour apporter une solution à la grande majorité des cas que pose l'exploration des ovaires, des testicules et des surrénales. Il n'est pas moins indispensable que, dans un laboratoire où l'on pratique le dosage de la folliculine, on dispose, à côté de notre technique, de la méthode chromatographique de BROWN. On devra y recourir dans trois circonstances bien définies:

1. Dans tous les cas d'hypofolliculinie où, pour une raison clinique, il est utile de préciser que le taux des œstrogènes est très faible; bien entendu, cette éventualité n'est pas fréquente, mais elle se rencontre lorsqu'il existe par exemple, une discordance entre les résultats de la cytologie vaginale ou utérine et la valeur des phénolstéroïdes totaux.

2. Dans tous les cas où le taux de folliculine, en dehors de la grossesse, dépasse 50 μg. Ces cas sont assez rares, lorsque le recueil des urines n'est pas fait au moment de l'ovulation ou après administration de HGC; aussi un taux élevé de folliculine trouvé à plusieurs déterminations successives doit-il faire penser, en dehors de la

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grossesse, à la possibilité d'une tumeur des gonades, des corticosurrénales ou d'un chorioépithéliome.

3. Dans tous les cas où il existe une discordance flagrante entre les résultats trouvés et la clinique.

Dans la pratique, le dosage des œstrogènes par chromatographie est utile dans moins de 3% des cas en dehors de la grossesse. Au cours de la grossesse, nous n'avons jamais rencontré de cas où le fractionnement des œstrogènes ait été nécessaire; il faut, bien entendu, veiller à ce qu'au moment du recueil des urines la malade n'absorbe pas de médicaments qui peuvent interférer dans le dosage (phénolphtaleïne des laxatifs par exemple).

Dans ce travail, nous n'avons abordé l'étude critique de cette méthode que du seul point de vue des critères analytiques; nous avons défini ses qualités, ses possibilités et ses limites. En fait, pour apprécier la valeur clinique d'une méthode et préciser la séméïologie nouvelle qu'elle apporte au clinicien, il est essentiel de se référer à des critères physio-pathologiques. Ces derniers ont en biologie clinique une importance qui est égale à celle des critères analytiques; nous les aborderons dans l'article suivant, où nous présenterons également des méthodes d'exploration dynamique que nous avons standardisées en vue de l'étude des fonctions endocriniennes du corps jaune et des testicules.

RÉSUMÉ

Nous présentons une méthode permettant le dosage des phénolstéroïdes totaux, c'est-à-dire en fait la somme de l'œstriol, de l'œstrone et de l'œstradiol-17β; cette méthode est exacte, précise et spécifique lorsque le taux des phénolstéroïdes est supérieur ou égal à 30 μg pour 1000 ml. Les résultats trouvés concordent avec ceux de la méthode chromatographique de BROWN. La récupération globale des œstrogènes ajoutés est pour la modalité avec saponification de 82% ($\sigma = 3\%$) pour l'œstriol et de 72% ($\sigma = 3\%$) pour l'œstrone. Au-dessous de 20 μg pour 1000 ml la méthode n'a pas une spécificité suffisante; les chromogènes interférents entraînent une erreur par excès qui, exprimée en équivalents d'œstrone, peut atteindre 10 μg pour 1000 ml.

Cette technique permet de résoudre la grande majorité des problèmes posés par la clinique car, d'une part, le dosage séparé des trois œstrogènes urinaires ne présente pas une valeur séméïologique plus grande que celui des phénolstéroïdes totaux, d'autre part, la recherche des taux de folliculine inférieurs à 20 μg pour 1000 ml ne présente en clinique qu'un intérêt mineur. Nous estimons que le dosage des phénolstéroïdes totaux peut être comparé à celui des 17-cétostéroïdes totaux et que le fractionnement chromatographique des œstrogènes ne doit être utilisé que dans des cas particuliers.

SUMMARY

PHENOLIC STEROIDS IN THE URINE. I. DETERMINATION

A method is proposed for the determination of the total phenolic steroids, *i.e.* the sum of œstriol, œstrone and œstradiol-17β. This method is accurate, precise and specific when the content of the phenolic steroids is 30 μg/1000 ml or more. The results are in agreement with those obtained by the chromatographic method of BROWN. The average recovery of added œstrogens amounts to 82% ($\sigma = 3\%$) for œstriol and 72% ($\sigma = 3\%$) for œstrone when the procedure with saponification is used. Below

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20 μg/1000 ml, the specificity of the method is no longer satisfactory because the interfering chromogens cause a positive error, which, expressed in equivalents of œstrone, may be as much as 10 μg per 1000 ml.

With this method it is possible to solve most of the problems met with in clinical practice, partly because the determination of the three urinary œstrogens separately is not of greater symptomatological value than that of the total phenolic steroids, and partly because the determination of folliculin contents of less than 20 μg/1000 ml is only of minor interest in clinical practice. The authors consider that the estimation of phenolic steroids is comparable to that of the total 17-keto-steroids, and that the chromatographic separation of the œstrogens should only be carried out in special cases.

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NOUVELLE MANIÈRE DE DÉTERMINER LE POIDS SPÉCIFIQUE DE L'URINE SUR UNE TRÈS PETITE QUANTITÉ

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Il est fréquemment impossible de recueillir une grande quantité d'urine dans les cliniques d'enfants et il est très difficile, faute d'une méthode simple, rapide et précise, de déterminer le poids spécifique de l'urine lorsqu'on dispose d'un faible volume. Les uromètres qu'on emploie le cas échéant permettent d'opérer sur 12–15 ml d'urine, avec une précision suffisante, si on dilue l'échantillon au tiers ou au quart. Nous proposons une méthode permettant de déterminer le poids spécifique à partir de 0.5–1.0 ml, et suffisamment précise pour l'usage clinique.

Nous nous sommes inspirés de la méthode de KIRKPATRICK ET KLING[1], suivant laquelle on introduit une goutte d'urine dans un mélange de tétrachlorure de carbone et de xylène (auquel l'urine n'est absolument pas miscible). La goutte d'urine doit se maintenir dans ce liquide, sans monter à la surface, ou descendre au fond. Afin d'obtenir ce résultat, il faut trouver la proportion relative de tétrachlorure de carbone et de xylène; ensuite on détermine le poids spécifique du mélange, qui est le même que celui de l'urine.

La base physique de notre méthode est la même, mais notre procédé est inverse. Nous avons en effet remarqué qu'une goutte de glycérol, introduite dans une colonne d'urine d'une certaine hauteur, ne se dilue pas immédiatement; si l'on colore le glycérol par un peu de bleu de méthylène, on peut facilement observer le mouvement de la goutte de glycérol dans l'urine, mouvement dû à la différence des poids spécifiques des deux liquides.

Solution de glycérol

Nous préparons une solution composée d'eau et de glycérol et de poids spécifique connu (1.025 de préférence). Cette solution est ensuite diluée de façon à obtenir toute une série de solutions standardisées dont le poids spécifique va de 1.001 à 1.025. Nous ajoutons à chaque solution quelques cristaux de benzoate de sodium comme bactériostatique, ainsi qu'une petite quantité de bleu de méthylène. Nous contrôlons ensuite le poids spécifique de ces solutions à l'aide d'un aréomètre: les deux substances supplémentaires n'ont presque aucune influence sur le poids spécifique. Nous versons les solutions dans des bouteilles munies de bouchons en caoutchouc percés d'une pipette pourvue d'une poire en caoutchouc.

Mode opératoire

Il existe deux procédés dont le choix dépend de la quantité d'urine dont on dispose: a) détermination directe, sans dilution de l'urine; b) détermination indirecte, après dilution.

a) Détermination directe. Nous versons dans une éprouvette la quantité d'urine nécessaire pour obtenir une colonne de liquide de 30 à 40 mm de hauteur: avec une

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éprouvette étroite (environ 8 mm de diamètre), il suffit d'habitude de 2 ml d'urine. Nous introduisons dans une pipette un peu de solution glycérolique de poids spécifique supérieur au poids supposé de l'urine et nous laissons tomber dans l'urine une goutte de cette solution, d'une hauteur de 0.5 cm au-dessus de la surface de l'urine. Si la goutte atteint le fond, nous introduisons dans la même urine une goutte d'une solution de poids spécifique plus faible; si celle-ci remonte à la surface, nous introduisons une goutte de poids spécifique plus élevé, et ainsi de suite jusqu'à ce que la goutte de la solution reste au milieu de l'urine, sans atteindre le fond ni remonter à la surface. Le poids spécifique de l'urine correspond alors au poids spécifique de cette dernière solution. On peut introduire quelques gouttes de solutions de différents poids spécifiques sans risquer de modifier le poids spécifique de l'urine.

b) Détermination après dilution. Si nous disposons d'une très petite quantité d'urine (0.5 ml au minimum), le procédé est le même, mais on dilue préalablement l'urine.

Si l'on dispose de 1 ml d'urine, on l'introduit dans une éprouvette et on ajoute 1 ml d'eau; puis on procède comme ci-dessus. On multiplie les deux derniers chiffres du résultats par la dilution d'urine, ici par 2.

Si l'on dispose d'une plus petite quantité d'urine, on ajoute 2 ou 3 fois plus d'eau et on fait de la même façon la mesure et le calcul du poids spécifique.

Influence de différents facteurs sur l'exactitude des résultats

1. Pour pouvoir se servir de cette méthode, il faut prendre en considération le facteur de constance du poids spécifique des solutions dans le temps. Les dilutions de glycérol peuvent être infectées par des microbes, se décomposer et, par suite, changer de poids spécifique. Ainsi qu'il résulte des nombres rapportés dans le Tableau I, le poids spécifique des solutions ne varie que très peu; cependant, il est nécessaire de procéder de temps à autre à des contrôles.

TABLEAU I

CONSTANCE DU POIDS SPÉCIFIQUE DES SOLUTIONS DANS LE TEMPS

Date de contrôle	*24/8*	*21/9*	*6/10*	*12/11*
1.003	1.003	1.003	1.003	1.0035
1.004	1.004	1.004	1.004	1.004
1.005	1.005	1.005	1.005	1.005
1.006	1.006	1.0055	1.006	1.006
1.007	1.007	1.0065	1.0075	1.007
1.008	1.008	1.0075	1.008	1.008
1.009	1.009	1.0085	1.0095	1.0095
1.010	1.010	1.009	1.0105	1.010
1.011	1.011	1.010	1.011	1.0115
1.012	1.012	1.011	1.012	1.012
1.013	1.013	1.012	1.013	1.0125
1.0135	1.0135	1.0135	1.014	1.0135

2. Nous avons déterminé le degré du changement du poids spécifique de l'urine, dû à l'addition des solutions standard. Nous avons ajouté à une quantité connue d'urine une solution standard, de poids spécifique différent de celui de l'urine, et nous

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TABLEAU II

VOLUME INITIAL DE L'URINE: 40 ml; VOLUME DE CHAQUE GOUTTE: 0.036 ml

Poids spécifique de la solution de glycérol ajoutée	1.003	1.003	1.015
Nombre de gouttes de solution de glycérol ajoutées	*Urine No. 1*	*Urine No. 2*	*Urine No. 3*
0	1.012	1.010	1.006
10	1.012	1.010	1.006
20	1.012	1.010	1.006
30	1.012	1.010	1.006
40	1.012	1.010	1.006
50	1.012	1.010	1.006
60	1.012	1.010	1.006
70	1.012	1.010	1.006
80	1.012	1.010	1.006
90	1.012	1.010	1.0065
100	1.012	1.0095	1.007

avons cherché la quantité nécessaire pour modifier sensiblement le poids spécifique de l'urine.

Il résulte des nombres rapportés dans le Tableau II qu'en ajoutant une solution dont le poids spécifique diffère notablement de celui de l'urine examinée, nous parvenons à modifier le poids spécifique de l'urine, à partir d'un volume de solution standard correspondant à 8% du volume de l'urine.

Si l'on applique ce résultat à la quantité d'urine mise en œuvre dans notre méthode, on voit que le poids spécifique de l'urine ne change qu'après addition de 5 gouttes d'une solution standard dont le poids spécifique diffère considérablement de celui de l'urine.

Or, pratiquement, seul le poids spécifique de la première goutte peut différer beaucoup de celui de l'urine; le poids spécifique des gouttes suivantes se rapproche de celui de l'urine examinée et la possibilité d'erreur devient de plus en plus faible.

3. L'examen a démontré qu'entre 8° et 32°, la température n'avait aucune influence sur la justesse de notre méthode. Il est cependant d'une grande importance que l'urine et la solution standard soient à la même température.

4. Les exemples suivants montrent l'influence de la dilution de l'urine sur la précision de notre méthode.

1er exemple

Poids spécifique de l'urine mesuré à l'aide de l'aréomètre: 1.024
Poids spécifique de l'urine mesuré d'après notre méthode: 1.024

Urine (ml)	*Eau (ml)*	*Dilution*	*Poids spécifique*	*Résultat calculé*
1	1	2	1.012	1.024
1	2	3	1.008	1.024
1	3	4	1.006	1.024
1	4	5	1.005	1.024
1	5	6	1.003	1.018

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2ème exemple

Poids spécifique de l'urine déterminé à l'aide de l'aréomètre: 1.007
Poids spécifique de l'urine déterminé d'après notre méthode: 1.007

Urine (ml)	*Eau (ml)*	*Dilution*	*Poids spécifique*	*Résultat calculé*
2	1	1.5	1.005	1.0075
1	1	2	1.0035	1.007
1	2	3	1.002	1.006

3ème exemple

Poids spécifique de l'urine déterminé à l'aide de l'aréomètre: 1.012
Poids spécifique de l'urine déterminé d'après notre méthode: 1.012

Urine (ml)	*Eau (ml)*	*Dilution*	*Poids spécifique*	*Résultat calculé*
1	–	1	1.012	1.012
2	1	1.5	1.009	1.013
1	1	2	1.006	1.012
1	2	3	1.004	1.012
1	3	4	1.0035	1.014

4ème exemple

Poids spécifique de l'urine déterminé à l'aide de l'aréomètre: 1.035

Urine (ml)	*Eau (ml)*	*Dilution*	*Poids spécifiques*	*Résultat calculé*
1	1	2	1.0175	1.035
1	2	3	1.013	1.0355
1	3	4	1.009	1.036
0.5	2	5	1.007	1.035
0.5	2.5	6	1.006	1.036
0.5	3	7	1.005	1.035
0.5	3.5	8	1.0045	1.036
0.5	4	9	1.0033	1.0315

CONCLUSIONS

Ces exemples nous permettent de déduire les conclusions suivantes:

a) lorsque le poids spécifique est petit, nous obtenons des résultats dont l'authenticité est indiscutable, à condition de diluer l'urine au tiers; si nous la diluons au quart, le résultat n'est qu'approximatif;

b) lorsque le poids spécifique est moyen (1.012 à 1.024), on peut diluer l'urine au quart avec de bons résultats;

c) lorsque le poids spécifique est plus grand (plus de 1.024), nous obtenons encore de bons résultats après dilution au 1/6e.

Nous avons exécuté dans notre laboratoire plus de 100 épreuves en nous servant parallèlement des deux méthodes: celle à l'uromètre et notre méthode stalagmométrique avec 2 variantes, sans dilution et avec dilution au demi. Les résultats obtenus

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sont identiques. Aussi, nous nous servons maintenant couramment de notre méthode pour déterminer le poids spécifique des urines qui nous sont envoyées en très petites quantités.

5. Quand on exécute la mesure, il faut prendre les précautions suivantes:

a) éviter la mousse à la surface du liquide formé du mélange d'urine et d'eau: ceci s'obtient en inclinant le tube à essai et en versant le liquide le long de la paroi du tube;

b) quand on ajoute les premières gouttes, il est préférable d'utiliser une solution standard relativement lourde, afin que les gouttes restent au fond du récipient;

c) les pointes des pipettes doivent être de la même largeur, afin que les gouttes soient, autant que possible, de la même dimension.

RÉSUMÉ

Nouvelle méthode ayant pour but la détermination du poids spécifique de l'urine sur un très petit volume. Elle est fondée sur l'observation du mouvement des gouttes de solutions standard colorées, de poids spécifique connu. Cette méthode permet d'opérer sur des quantités aussi faibles que 0.5 ml. Elle présente l'avantage d'être simple, rapide et précise et elle possède donc les qualités requises pour être utilisée dans les laboratoires de cliniques pédiatriques.

SUMMARY

DETERMINATION OF THE SPECIFIC GRAVITY OF URINE IN VERY SMALL QUANTITIES

A new method is presented for the determination of the specific gravity of very small quantities of urine. It is based on the movement of drops of stained standard solutions of known specific gravity in the urine. By this method quantities as small as 0.5 ml can be estimated. Owing to its simplicity, rapidity and accuracy, the method possesses all the qualities necessary for use in the laboratory of a pediatric clinic.

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THE DETERMINATION OF THE SODIUM SALT OF 5-IODOSALICYLIC ACID AND ITS METABOLITES IN URINE

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INTRODUCTION

In a former publication[1] a method for the determination of sodium 5-iodosalicylate (5-I-S-Na) in serum was described. Ultraviolet light absorption was used. In the work reported in the present communication, the determination of 5-I-S-Na in urine with the use of the same principle is studied. At the same time determinations of total iodine in the urine were carried out to study the breakdown of 5-I-S-Na.

METHODS

Determination of 5-I-S-Na in urine

It was found that 5-iodosalicylic acid (5-I-S) was present in urine partly in a free state (A), and partly in a conjugated form (B). These two fractions A and B were determined separately.

A. Determination of free 5-I-S. 10 ml urine were acidified with sulphuric acid (10 *N*) at pH 1. 20 ml CCl_4 were added and the mixture was shaken mechanically for 1 h. Care was taken to exclude light by wrapping the vessel in black paper to avoid destruction of 5-I-S. The mixture was separated by centrifuging. The urine layer was removed and 7 ml of this layer shaken again with 7 ml CCl_4. The CCl_4 layer of the first extraction (I) was diluted 1 : 10. The CCl_4 layer of the second extraction (II) was not diluted. The concentration of 5-I-S in each portion (I and II) was determined by the spectrophotometric method with a Beckman D.U. The concentration was calculated as follows: Conc. urine = 20 × value I + value II.

B. Determination of total 5-I-S-Na (free and conjugated). 10 ml urine and 1 ml HCl 38% were boiled for 30 min. It was found that a prolongation of the boiling did not give a higher yield of 5-I-S. After cooling the pH was adjusted to 1. The determination was then carried out in the same manner as in A.

A single determination at the maximum of absorption (330 mμ) can be used in pure solutions, but in urine the possibility of unreliable results owing to the considerable blank values has to be considered. Therefore another method which is independent of the blank values has been carried out for comparison. This method has been described in principle by ALLEN[2], who has shown that the value

$$AB = E_{330} - \frac{E_{310} + E_{350}}{2}$$

is independent of the blank values at 310, 330 and 350 mμ if these values are in a straight line (Fig. 1). It was shown that this assumption was correct in the present investigation. In Fig. 2 are given the standard curves of 5-I-S in CCl_4 for the determinations at 330 mμ and for the determinations at 310, 330 and 350 mμ.

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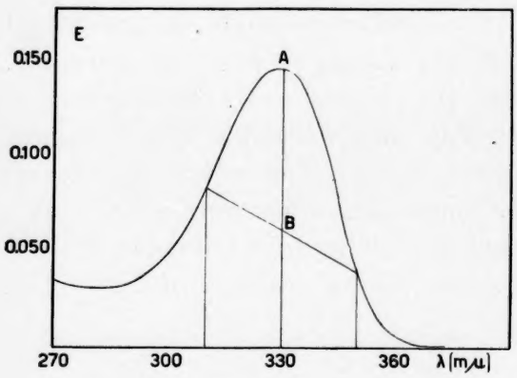

Fig. 1. The absorption curve of 5-I-S in CCl_4. The value of AB is independent of the blank values in the urine at 310, 330 and 350 mμ, if these values are on a straight line.

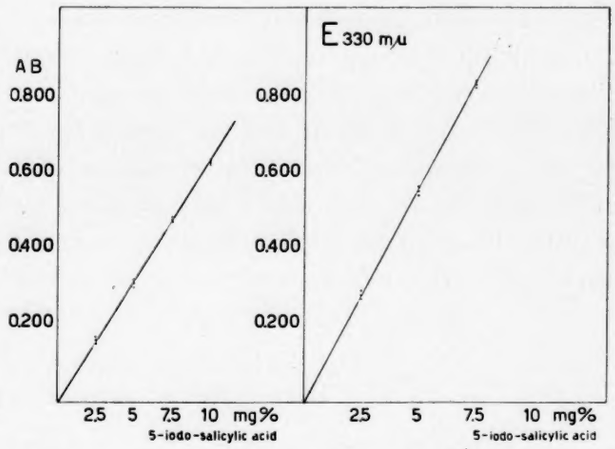

Fig. 2. The standard curves of 5-I-S in CCl_4 for the single extinction at 330 mμ and for the values of AB.

Control of the method

In a series of normal urines the standard procedure for 5-I-S as described above was carried out and the blank values with a single reading at 330 mμ and the corrected values with the method of ALLEN were determined (Table I).

The single reading at 330 mμ gave a blank value varying between 0.3 and 3 mg% in fresh urine, while blank values between 3.5 and 6.3 mg% were found in boiled urine. The method of ALLEN reduced the blank values to maximal 0.09 mg% in fresh urine and 0.5 mg% in boiled urine.

In the next experiments 5-I-S-Na was added to the urine. In the fresh urine (Table II) good recoveries were obtained both with the single reading and the cor-

TABLE I DETERMINATION OF "5-IODOSALICYLIC ACID IN" NORMAL URINES

Urine	*Fresh urine*		*Boiled urine*	
	Determ. at 330 mμ (mg%)	*Determ. at 310, 330, 350 mμ* (mg%)*	*Determ. at 330 mμ (mg%)*	*Determ. at 310, 330, 350 mμ* (mg%)*
1	0.4	—0.04	3.5	—0.5
2	0.8	0.02	3.8	0.5
3	1.9	0.04	4.9	0.1
4	3.0	0.09	6.3	—0.5
5	0.3	0.03	5.7	—0.3
6	1.0	0.03	5.5	0.5
Mean value	1.2	0.03	5.0	0.3

* Method of ALLEN.

TABLE II RECOVERY OF 5-I-S-Na FROM FRESH URINE

Urine	*50 mg% added*		*Urine*	*100 mg% added*	
	330 mμ (mg%)	*310, 330, 350 mμ* (mg%)*		*330 mμ (mg%)*	*310, 330, 350 mμ* (mg%)*
1	50.8	50.1	5	104.8	104.2
2	50.7	49.8	6	106.1	106.3
3	48.1	49.4	7	103.4	102.3
4	51.5	52.4	8	99.6	98.8

* Corrected method.

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rected method, and the differences between both methods were slight. In the boiled urine (Table III) there was a larger variation in the values which were obtained. The single reading values were in general at a somewhat higher level. The mean value found after addition of 100 mg% was 102.6 mg. The values found after addition of 50 mg% showed a considerable variation (38.9–52.9 mg%). The mean value found after addition of 24.5 mg% was 26.3 mg%. The corrected values were inequal at a slightly lower level with a mean value of 95 mg% after addition of 100 mg%, of 44.1 mg% after addition of 50 mg%, and of 21.1 mg% after addition of 24.5 mg%.

TABLE III RECOVERY OF 5-I-S-Na FROM BOILED URINE
(10 ml urine boiled 30 min with 1 ml HCl 38%)

Urine	*24.5 mg% added*		Urine	*50 mg% added*		Urine	*100 mg% added*	
	330 mμ (mg%)	*310, 330, 350 mμ* (mg%)*		*330 mμ (mg%)*	*310, 330, 350 mμ* (mg%)*		*330 mμ (mg%)*	*310, 330, 350 mμ* (mg%)*
1	23.5	21.2	7	48.6	43.9	14	100.2	94.3
2	27.6	25.4	8	52.9	49.3	15	104.6	99.9
3	25.3	20.9	9	47.8	43.4	16	102.8	94.8
4	26.8	20.6	10	45.8	41.6	17	98.2	90.9
5	27.2	16.6	11	38.9	42.0	18	104.5	96.7
6	25.7	22.1	12	52.0	47.3	19	100.1	91.5
			13	52.4	44.7	20	105.4	97.7
						21	105.3	95.2
Mean value	26.3	21.1	Mean value	48.3	44.1	Mean value	102.5	95.1
Deviation	+1.8	—3.4	Deviation	—1.7 +0 **	—5.9	Deviation	+2.5	—4.9

* Corrected method. ** [1] Urine no. 11 not included.

These figures suggest that the boiling procedure leads to a slight destruction of 5-I-S independent of the amount of 5-I-S, for the values of 25 mg%, 50 mg% and 100 mg%. To clear up this point the absorbtion values of pure solutions of 5-I-S-Na in water were determined before and after boiling. Slightly lower values were found in the boiled solutions (97.3–99.4 mg%). On the other hand, boiling increases the blank values, which causes too high values in the single reading at 330 mμ. It is probable that the most correct values can be obtained in the boiled urine by using the correction method of ALLEN and adding 4.7 mg% for correction of destroyed 5-I-S. In practice however the variations are so small that even a single reading at 330 mμ gives satisfactory results, if no special interfering substances are present (*e.g.* salicylic acid).

Determinations in the urine of patients who received 5-I-S-Na orally

The method described above was applied to patients who were treated with 5-I-S-Na (in several patients with acquired haemolytic anaemia). Table IV shows that a close agreement exists between the single determination at 330 mμ and the corrected values with the method of ALLEN. In general the corrected values were slightly lower than the 330-mμ values, which was also found in the recovery experiments (Table III).

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TABLE IV DETERMINATION OF 5-I-S-Na IN THE URINE AFTER ORAL ADMINISTRATION

Urine	Concentration free 5-I-S-Na		Concentr. bound 5-I-S-Na		Received daily 5-I-S-Na (g)	Recovery daily 5-I-S-Na	
	330 mμ (mg%)	310, 330, 350 mμ (mg%)	330 mμ (mg%)	310, 330, 350 mμ (mg%)		330 mμ (g)	310, 330 350 mμ (g)
1	8.0	7.1	162	157.8	3	2.5	2.4
2	9.3	8.5	174.7	169.7	5	3.6	3.5
3	7.4	6.6	191.6	185.5	4	2.8	2.7
4	6.3	4.7	197.4	196.2	4	2.8	2.7

This agreement suggests that the values which were obtained correctly represent the 5-I-S as such, and are not markedly influenced by metabolites of 5-I-S. The values show that only about 3% of the 5-I-S-Na is present in the free state, and the rest is in the conjugated form. Furthermore it will be seen that a considerable amount (16–30%) of the ingested 5-I-S is not recovered from the urine. This finding made it desirable to study the total iodine content of the urine. In the literature two methods are described.

The first method[3] utilizes the alkaline oxidation with $KMnO_4$ with formation of IO_3^- The excess of $KMnO_4$ is eliminated by addition of alcohol. The solution is freed from the precipitated MnO_2 by filtration and from HNO_2 by boiling with acetic acid. The final solution to which KI in solid form is added is titrated with sodium thiosulphate. This method gives only a recovery of 90% of added 5-I-S-Na as iodine.

In the second method[4] which also uses $KMnO_4$ in alkaline solution the excess $KMnO_4$ and MnO_2 are eliminated by $NaNO_2$, while the $NaNO_2$ is eliminated by urea. The final solution is again titrated with sodium thiosulphate after addition of KI. This method, as described, gave very unreliable results.

However, much better results were obtained if care was taken to avoid an excess of $NaNO_2$. The modified method is given here: 5 ml urine, 1 ml 20 *N* H_2SO_4 and 10 ml of a saturated solution of $KMnO_4$ are mixed and placed on a steam bath for 30 min. The mixture is shaken repeatedly to avoid the adherence of MnO_2 to the wall of the glass vessel. The solution is then brought to and kept at a temperature of 90° and a solution of $NaNO_2$ (7%) is slowly added from a burette until the MnO_2 has just disappeared. Any excess of $NaNO_2$ should be avoided. 4 ml of urea (30% sol.) are then added and the whole is cooled. The solution is titrated with $Na_2S_2O_3$ solution (0.03 *N*). In a few cases the end point was uncertain. These determinations were discarded.

The results obtained in urine after addition of 5-I-S-Na are given in Table V. It will be seen that this modified method gives satisfactory results.

TABLE V DETERMINATION OF ADDED 5-I-S-Na IN THE URINE BY THE MODIFIED METHOD

Urine	Quantity of 5-I-S-Na added		
	100 mg% (mg%)	200 mg% (mg%)	300 mg% (mg%)
1	104	202	300
2	102	205	303
3	103	205	303
4	102	207	301

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Determination of 5-I-S-Na and total iodine in the urine of patients who received 5-I-S-Na orally

The results of these determinations are given in Table VI. The absorption values of columns 2 and 3 are in agreement with the former determination. The values for total iodine expressed as 5-I-S-Na are higher than those obtained with the direct determination of 5-I-S-Na, although even for total iodine no quantitative recovery from the injected amount is obtained (recovery 70–85%). In the faeces of some patients only traces of iodine were found (50 mg daily, expressed as 5-I-S-Na)by the method of GERARD AND RAUNET[4]. These results indicate that 5-I-S-Na is broken down in the body. Inorganic iodine could not be demonstrated in these urines.

TABLE VI DETERMINATION OF 5-I-S-Na AND TOTAL I_2 AFTER ORAL ADMINISTRATION OF 5-I-S-Na

Urine	*Concentration of total 5-I-S-Na*		*Concentr. total I calcul. as 5-I-S-Na*	*Received daily 5-I-S-Na*	*Recovery of 5-I-S-Na*		*Recov. I_2 calcul. as 5-I-S-Na*
	330 mμ (mg%)	*310, 330, 350 mμ (mg%)*	*(mg%)*	*(g)*	*330 mμ (g)*	*310, 330, 350 mμ (g)*	*(g)*
1. K	180.3	175.8	234	4	2.5	2.4	3.2
2. B	150.1	146.9	200.4	4	2.6	2.5	3.4
3. I	163.4	151.3	190	4	2.6	2.4	3.0
4. V	94.8	90.5	114	2	1.1	1.1	1.4

SUMMARY

A method for the determination of the sodium salt of 5-iodosalicylic acid (5-I-S-Na) and total iodine is described. The method for the determination of 5-I-S-Na is based on the ultraviolet light absorption of this substance. Control experiments with the method for the determination of 5-I-S-Na and total iodine showed a satisfactory recovery for added 5-iodosalicylic acid (5-I-S). The boiling of the solution with hydrochloric acid necessary for the hydrolysis of conjugated 5-I-S caused only a slight loss of this acid. It was found that the values for 5-I-S correctly represent this substance, and that only about 3% of this acid was present in a free state. The rest was in a conjugated form. The presence in the urine of an excess of iodine, as compared with total 5-I-S, indicated the presence of metabolites of this acid. Inorganic iodine was not found.

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SHORT COMMUNICATIONS

A sensitive spectrophotometric method for adrenaline and noradrenaline*

Colorimetric methods for adrenaline which are based on oxidation to the red compound, adrenochrome, are relatively insensitive because the molecular extinction coefficient of this compound at its maximum in the visible spectrum is relatively low. These methods require between 20 and 200 μg of adrenaline[1]. Fluorometric methods are much more sensitive, requiring less than 1 μg of the medullary hormones. However, these methods are attended by various technical difficulties and require rigid control to maintain reasonable accuracy. The present method is intermediate in sensitivity between the fluorometric methods and the colorimetric methods mentioned above, being useful with the amounts usually available following paper chromatographic separation, *i.e.* between 1 and 20 μg. It retains the simplicity of colorimetric methods, although it cannot be classed as a colorimetric method since it measures the absorption of light of a wave length slightly below the visible range. In this region the light source may be either a tungsten or a hydrogen lamp. The cuvettes may be either glass or quartz. The increased sensitivity over the methods based on determination of adrenaline as adrenochrome (or iodoadrenochrome) is made possible by use of a derivative or transformation product (I) of adrenochrome which has a much higher absorption peak than the adrenochrome peak in the visible range.

Adrenaline is determined by oxidation to adrenochrome (or iodoadrenochrome), then converted to a compound (I) which is remarkably stable at pH 5 and has a strong absorption at 347 mμ. It is apparently identical with intermediate (V) of HARLEY-MASON[2] for which he has proposed a zwitterionic structure. Both compounds absorb maximally at 347 mμ and both are readily converted to 3,5,6-trihydroxy-1-methylindole (adrenolutin) on addition of alkali.

Similarly, noradrenaline is determined by oxidation to noradrenochrome (or iodonoradrenochrome) followed by conversion to the analogous compound (II) absorbing at 350 mμ.

Using the procedure outlined below, adrenaline or noradrenaline, when separated chromatographically, may be estimated within an error of less than 3% when 20 μg or more are available for analysis. With 10 μg the error is within 5%. Although perceptible readings can be obtained with as little as 1 μg, the % error is much higher.

The reagents are the same as those of the modified VON EULER AND HAMBERG method described earlier[3], except that 1 *N* acetate is used rather than 0.1 *N*. The increased concentration of acetate facilitates formation of (I) from adrenochrome or iodoadrenochrome. The latter are formed by oxidizing adrenaline with iodine followed by removal of excess iodine with thiosulfate. The reaction products of iodine and thiosulfate apparently include a small amount of the reducing compound, hydrosulfite (dithionite), which is capable of producing the HARLEY-MASON reaction under these

* This investigation was supported in part by research grant H-3231 from the National Heart Institute, U.S. Public Health Service.

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conditions. The reducing activity of thiosulfate alone does not produce this reaction, as shown by the failure of sodium thiosulfate to yield (I) when added to a solution of adrenochrome* in acetate buffer. The reaction products, potassium iodide and sodium tetrathionate**, when added to adrenochrome or iodoadrenochrome*** also give negative results. However, addition of the premixed iodine and thiosulfate solutions does give rise to (I) on standing.

Procedure

Test spots containing 2 to 25 μg of adrenaline or noradrenaline on filter paper are cut out and placed in 10-ml flasks. To each is added 2 ml of 1 *N* acetate buffer, pH 5.0. The flasks are placed in a water bath at 30° and 0.4 ml of 0.1 *N* iodine solu-

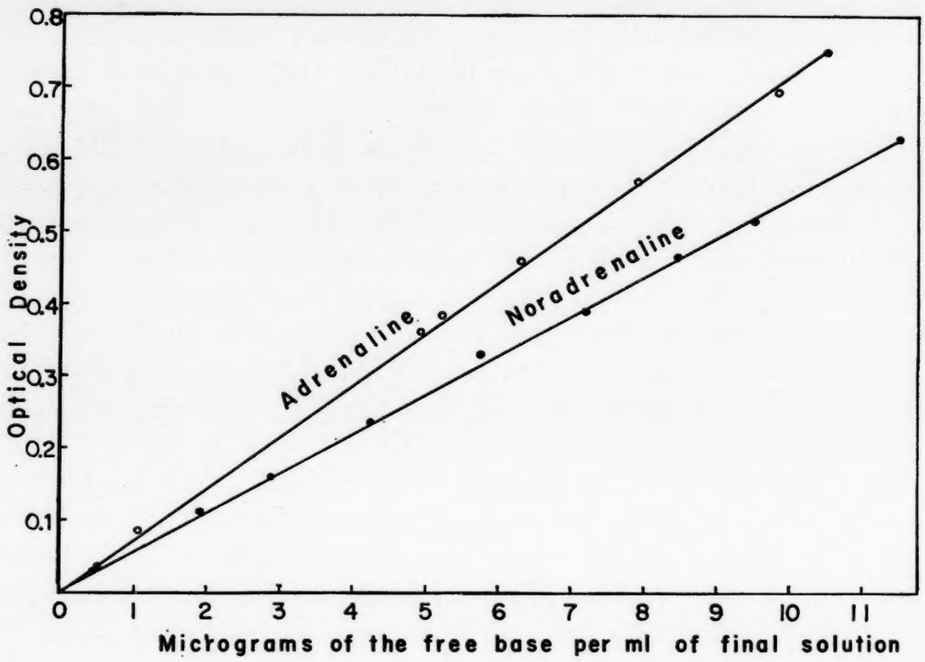

Fig. 1.

tion[3] is added. After mixing and standing 1 min for adrenaline and 5 min for noradrenaline, 0.9 ml of 0.05 *N* sodium thiosulfate is added. The mixture is shaken until the iodine color disappears. It is allowed to stand in stoppered flask at room temperature for 18 to 20 h, transferred to cuvette, and read against a reagent blank prepared simultaneously. Wave length 347 mμ is used for adrenaline and 350 for noradrenaline. A sensitive instrument such as the Beckman DU Spectrophotometer is used, with the standard 1-cm cuvette. The calibration curves shown here were prepared by analyzing 2-ml portions of standard solutions of adrenaline bitartrate or noradrenaline hydrochloride freshly prepared in 1 *N* acetate buffer, pH 5. Essentially the same results are

* We are grateful to Dr. NORMAN BARSEL of International Hormones, Inc. for kindly supplying crystalline adrenochrome freshly prepared and to Dr. J. D. CHANLEY, Mt. Sinai Hospital, for his suggestion with regard to obtaining the freshly prepared compound.

** Prepared by a method recommended by Dr. JOHN H. SPEER of G. D. Searle & Co.

*** Prepared by the method of SOBOTKA AND AUSTIN[4].

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obtained when the standards are introduced on filter paper and eluted as described above.

Further studies concerning the structure of (I) are in progress.

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* Medical Student Research Fellow, U.S. Public Health Service, Summer 1958.

Adenosine and inosine nucleotides content of human placenta

Recently we described methods[1] for the separation and determination of adenosine and inosine nucleotides and nucleosides in microgram quantities in tissues. This has allowed us to study the content of these substances in human placenta.

Small pieces were excised from the placenta of a normal pregnancy and delivery immediately after the expulsion, rapidly washed from blood under running tap water and frozen in acetone–dry ice mixture. The samples were then homogenized at 0° in 2 vol. (wet wt.) ice-cold 20% trichloroacetic acid. The homogenate was centrifuged at —2° and an aliquot of the supernatant analyzed by paper chromatography as previously described[1]. The quantitative determination of adenosine and inosine nucleotides was done by the orcine reaction after elution of the spots.

Results are given in Table I.

TABLE I

ADENINE AND INOSINE NUCLEOTIDE CONTENT OF HUMAN PLACENTA*

	Na_4ATP	Na_2ADP	Na_2AMP	Na_2IMP
μg/100 g wet weight	46.5 ± 6.4 $n = 9$	42.4 ± 6.8 $n = 8$	37.7 ± 2.4 $n = 7$	27 ± 4.7 $n = 9$

* AMP, ADP and ATP = adenosine mono-, di- and triphosphate; IMP = inosine monophosphate.

It can be seen that for the content in ATP and ADP, variability is much greater than for AMP. This can partly be due to the presence in placenta of a strong ATPase, ADPase and adenylate kinase activity, which will be described in detail elsewhere.

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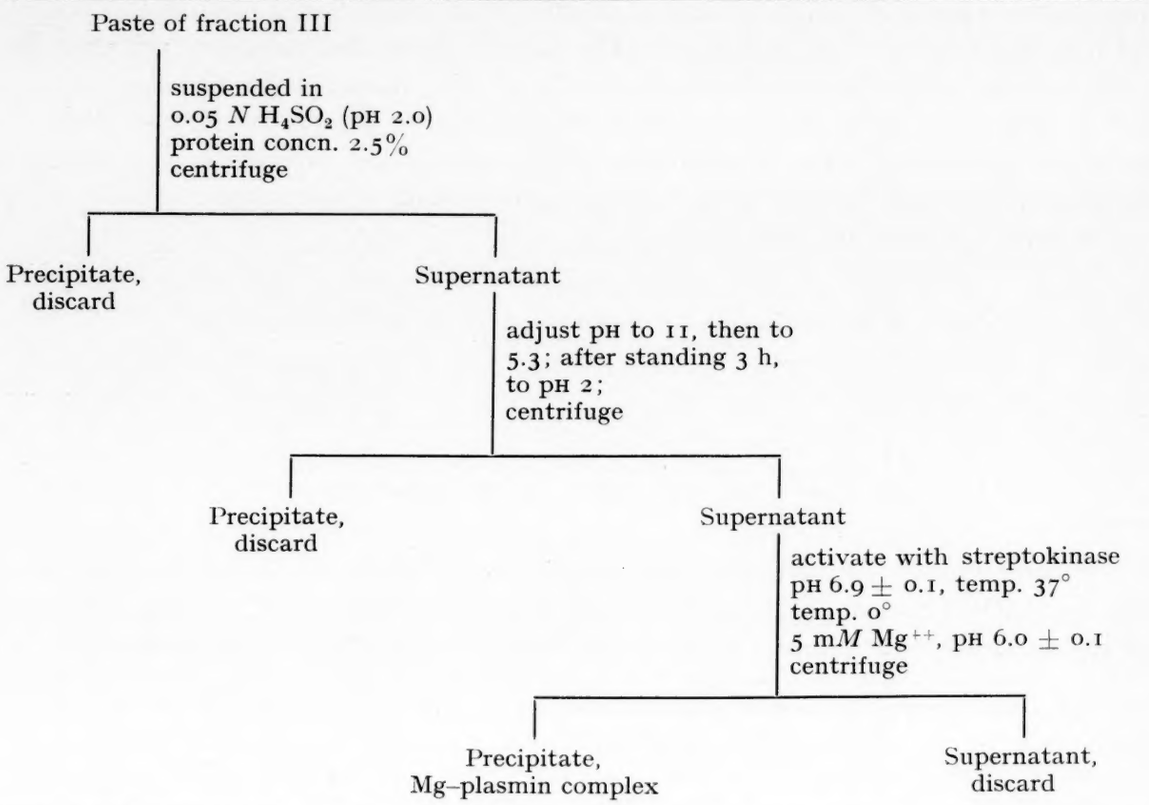
The use of Mg^{++} for the isolation and purification of human plasmin

HACKETHAL's and BIERSTEDT's[1] findings concerning the activating effect of Mg^{++} salts on the fibrinolytic system *in vivo* and the use of these salts in the therapy of thrombophlebitis and phlebothrombosis by HEINRICH[2], induced us to use the activating property of Mg^{++} for the preparation of a fibrinolytic enzyme plasmin for therapeutic purposes. Our aim was to prepare a complex Mg–plasmin which would combine the properties of both ingredients. It was found that under certain conditions Mg^{++} forms an insoluble complex with plasmin, which can be isolated from a solution of plasma proteins by removing the other proteins present.

Fraction III, obtained by ethanol fractionation of human plasma by a modification of COHN's method 10*, was extracted in 0.05 *N* H_2SO_4[3] at a protein concentration of 2.5%. After 1 h stirring the solution was centrifuged. The pH of the supernatant was adjusted to 11 (0.5 *N* NaOH) and after 5 min to pH 5.3 (0.5 *N* HCl). After 3 h standing, the pH was adjusted to 2.0 (0.5 *N* HCl), the solution was mixed and again centrifuged. From the solution obtained after activation with streptokinase (pH 6.9), an insoluble Mg–plasmin complex was isolated after precipitating with a 0.5 *M* solution of $MgCl_2$ up to a concentration of 5 m*M* Mg^{++} at pH 6.0 ± 0.1.

TABLE I

SCHEME FOR THE ISOLATION AND PURIFICATION OF HUMAN PLASMIN



* Fraction III was isolated according to a modification of COHN's method 10, by extraction of fraction II + III at pH 5.0 and ionic strength 0.015, with 8% alcohol.

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A scheme of this process is given in Table I. It was found that the pH at which the precipitation of Mg–plasmin takes place is dependent on the ionic strength of the solution (Fig. 1) and that the complex formation is specific under the conditions indicated in Table I. After 2 h standing at O°, the precipitate is centrifuged, dissolved and lyophilized after the addition of a minute amount of 0.5 *N* NaOH. By this rapid and simple method 90% of plasmin can be isolated from a solution in which the other human plasma proteins (albumin and globulins) are present.

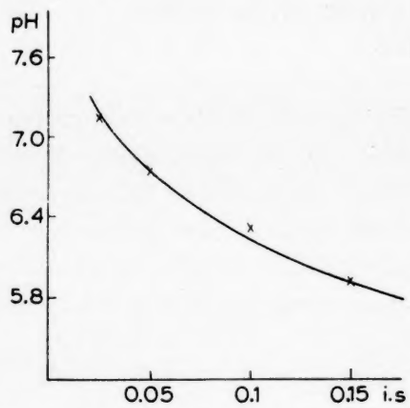

Fig. 1. Effect of pH and ionic strength on the solubility of the Mg–plasmin complex.

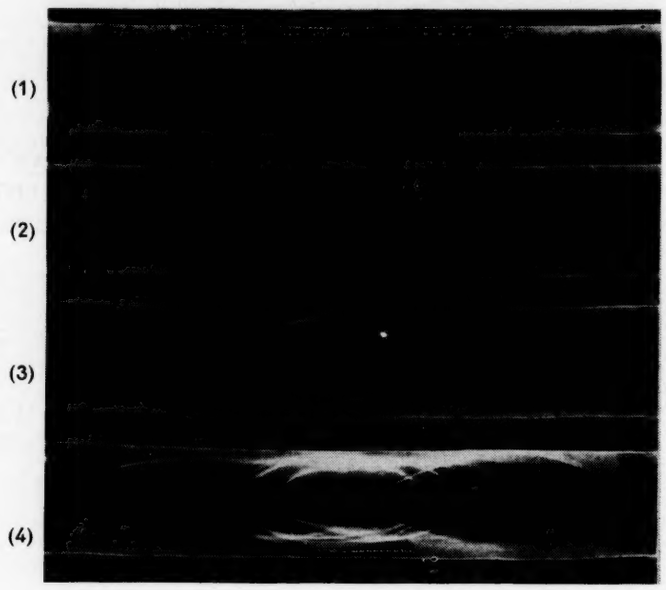

Fig. 2. Immunoelectrophoresis of the preparations mentioned in Table II. (1) Preparation 1. (2) Preparation 2. (3) Preparation 3. (4) Plasma.

65–70% of fibrinolytic activity as determined by a modification of FERGUSON's[4] method for fibrinogen[5] could thus be obtained from the total plasminogen content of fraction III. The specific content of units/1 mg *N* ranged between 160,000–200,000. The preparation was analysed by paper electrophoresis, which gave one single zone in the region of the β-globulins, and by immunoelectrophoresis, which gave 2 zones of β-globulins of the same or nearly the same mobility. In the final product the Mg^{++} and protein content were determined and their ratio was found to be constant within certain limits (Table II).

By this simple method an active preparation of Mg–plasmin can be obtained,

TABLE II

YIELD OF PLASMIN FROM FRACTION III

Preparation	*Activity content of fraction III (units/1,000 ml of plasma)*	*Yield (units/1,000 ml of plasma)*	*Spec. activity (units/mg N)*	*Content of Mg^{++}/g protein*
1	$680 \cdot 10^3$	$405 \cdot 10^3$	$167 \cdot 10^3$	0.0096
2	$625 \cdot 10^3$	$418 \cdot 10^3$	$189 \cdot 10^3$	0.0072
3	$670 \cdot 10^3$	$464 \cdot 10^3$	$162 \cdot 10^3$	0.0120

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which combines the fibrinolytic activity of plasmin and the activating effect of Mg^{++} on the fibrinolytic system *in vivo*.

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Application of an anthrone method for the estimation of glucose in a synthetic medium used for growing chick embryonic fibroblast tissue *in vitro*

Attempts are being made in this laboratory to study the relation between glucose uptake and the proliferation of animal tissues grown in a synthetic medium. While searching for a suitable method to estimate microquantities of glucose in the medium, it has been found that the anthrone method described by YEMM *et al.*[1] can be conveniently used. Details of experiments which indicate the validity of this method for measuring microquantities of glucose in fresh and contact media are recorded in this communication.

TABLE I

DETERMINATION OF GLUCOSE IN STANDARD GLUCOSE SOLUTION AND FRESH AND USED M150 MEDIA

	Standard glucose solution		*Fresh M150 medium*			*Used M150 medium*		*Used M150 medium (fermented)*	
Amount of the sample (ml)	*Klett reading (540 mμ)*	*Glucose present (μg)*	*Klett reading (540 mμ)*	*Glucose observed (μg)*	*content theoretical (μg)*	*Klett reading (540 mμ)*	*Glucose content observed (μg)*	*Klett reading (540 mμ)*	*Glucose content observed (μg)*
0.001	3.0	1.0	3.0	1.0	1.0	2.7	0.90	–	–
0.002	6.0	2.0	6.0	2.0	2.0	5.4	1.80	–	–
0.004	12.0	4.0	12.0	4.0	4.0	10.8	3.60	–	–
0.010	30.0	10.0	30.0	10.0	10.0	27.5	9.16	–	–
0.020	60.0	20.0	60.0	20.0	20.0	54.0	18.00	–	–

M150 medium[2] which contains 100 mg glucose per 100 ml was used for the experiments. Samples were studied of the fresh medium, and the medium in which chick embryonic fibroblast tissue was grown for six days. Various amounts of these two media were taken and their glucose content estimated by the method of YEMM *et al.*[1] Care was taken to see that the sugar tubes were completely dry, and the anthrone reagent was prepared in glass distilled water every day; the colour of the reagent was yellow.

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The results are given in Table I. The results of recovery experiments carried out on the used medium are given in Table II.

TABLE II

RECOVERY OF GLUCOSE ADDED TO USED M150 MEDIUM

Amount of the medium taken (ml)	*Glucose added (μg)*	*Klett reading (540 mμ)*	*Glucose present observed (μg)*	*recovery (%)*
0.010	–	27.5	9.16	–
0.010	10.0	58.0	19.16	100.0
0.010	20.0	87.0	28.84	99.2

Various amounts of used M150 medium were fermented using *S. cerevisiae*. The anthrone method was applied when all the glucose was fermented and gave a negative test for glucose. This shows that the method is specific for glucose.

It is thus evident that the method can be used to estimate microquantities of glucose present in the synthetic medium. The method is being used with success in this laboratory to estimate sugar uptake during tissue proliferation in various culture media.

The authors' thanks are due to the Indian Council of Medical Research for having sponsored the research project.

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Isolation and partial purification of T-agglutinin deriving from human plasma

Studying the virus-receptors of red blood cells[1] a more detailed examination of the phenomenon of T-agglutination(panagglutination[2]) appeared to be necessary. An attempt was made therefore to obtain concentrated and purified preparations of T-agglutinin from human citrate-plasma by means of cold ethanol fractionation.

Human AB-plasma containing neither isoagglutinins nor irregular agglutinins was used. Quantitative T-agglutinin determinations were performed by slide-agglutination tests in twofold dilution series of the plasma and of the fractions obtained, by means of washed human red blood cells pretreated with $M/500$ KIO_4. Isoagglutinin was estimated by slide-agglutination tests using red blood cells. The pH of the solutions was determined by means of a glass-electrode; the SCHULEK-VASTAGH modification[3] of the micro-Kjeldahl method was employed for N-determinations.

To prepare T-agglutinin solutions of the concentration and purity required, the following method has been adopted as satisfactory:

The pH of the plasma was adjusted to 7.2 by adding 0.2 *M* acetate buffer, diluted to 1 : 2 with saline and chilled to 4°. More of chilled acetate buffer (ionic strength 0.09, pH 6.7) containing 50% v/v ethanol was then added bringing the final ethanol concentration to 14.3% v/v. The temperature was simultaneously lowered to —4°. The resulting precipitate (P-I) contained all the T-agglutinin together with a number of other proteins.

After sedimenting the precipitate in a refrigerated centrifuge with 2500 r.p.m. at —4°, the supernatant (S-I) was discarded and the precipitate stirred for 30 min at 0° with chilled acetate buffer (ionic strength 0.08, pH 5.2) of half the volume of the starting plasma. The bulk of the T-agglutinin being thus redissolved, the mixture was centrifuged with 2500 r.p.m. at 0° for 30 min and the precipitate (P-Ia) discarded.

To the supernatant (S-II), chilled acetate buffer (ionic strength 0.07, pH 6.3) containing 15% v/v ethanol was added up to 30% of the initial plasma volume. The T-agglutinin and some other proteins were precipitated (P-II). After centrifuging with 2500 r.p.m. at —4.0° for 30 min and discarding the supernatant (S-III), the precipitate (P-II) was redissolved in saline of 1/10th of the initial plasma volume. This final solution showed T-agglutinin titers up to 6–8 times greater than that of the original plasma, whereas the protein content did not exceed 2–3% of that of the starting material.

TABLE I

PANAGGLUTININ AND T-AGGLUTININ TITERS OF PLASMA FRACTIONS AND SUPERNATANTS

	Plasma	*S–I*	*P–I/a*	*S–II*	*P–II*	*S–III*
T-agglutinin titers	1/8	ø	1/4	1/2	1/32	ø
Isoagglutinin titers	1/64	1/16	1/64	1/32	1/64	o

When comparing the method described above to that described by PILLEMER *et al.*[4] for concentrating isoagglutinins, it can be concluded that the solubility data of the T-agglutinin differ markedly from those of the isoagglutinin. In order to obtain

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additional evidence of this difference, T-agglutinins were purified by the method described above, using however plasma containing typed isoagglutinin. The fractions and supernatants were each examined both for T-agglutin and isoagglutinin content.

According to the results presented in Table I, the supernatants containing no T-agglutinins still contained significant amounts of isoagglutinins, suggesting the existence of differences between the physicochemical properties of the T-agglutinins and the isoagglutinins.

The technical assistance of Mr. B. VARGA and Mrs. A. OLLÉ is gratefully acknowledged.

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BOOK REVIEW

Thannhausers Lehrbuch des Stoffwechsels und der Stoffwechselkrankheiten, edited by N. ZÖLLNER, Georg Thieme Verlag, Stuttgart, 1957, 1040 pages, 230 figures, 124 tables, DM 127.—.

In 15 chapters this book covers general metabolism, intermediate metabolism, metabolism of carbohydrates, proteins, mucopolysaccharides, fats and fatty acids, lipids, steroids, bile acids, haem pigments, blood coagulation, iron, calcium, phosphate, water and electrolytes. Fifteen contributors from various countries have joined forces in supplying authoritative information on their special fields.

The basic idea behind this book is that the medical practitioner should have a fairly extensive knowledge of biochemistry. The reviewer considers that though this may be necessary in Germany where there are not many clinical chemists available, it is, nevertheless, impractical to compile a textbook on the basis of such an ideal. An attempt is made to combine in one chapter too many widely differing topics, *e.g.* a single chapter deals with the pure organic chemistry of the bile acids and the elucidation of their structure, the problems connected with their analysis in the clinical chemical laboratory, and their physiology, pathology and clinical significance. A real synthesis of these topics is impossible and, moreover, unnecessary. As a result of this policy many problems are not treated as expertly as in the handbooks of the various disciplines. In many chapters there is a lack of balance between the chemical, physiological and clinical aspects of the subject, only one of these being treated excellently, namely that coinciding with the author's own special field. Some of the chapters are outstanding, *e.g.* that on the metabolism of the proteins, especially the clinical part. It is a fine example of how a small amount of chemical information can be combined with a lot of clinical information to provide clinicians with all they need to know.

The reviewer considers that despite these drawbacks the book constitutes a magnificent reference work, which demands our respect. For the clinical chemist it should be very valuable as a work to be consulted on the many problems that arise in hospital laboratory practice.

J. C. M. VERSCHURE (Utrecht)

ANNOUNCEMENT

7TH COLLOQUIUM, ST. JANS HOSPITAL, BRUGES (BELGIUM)

1st–3rd May 1959

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1. *Special Topics*

   a. The nutritive value of proteins and protein deficiency.

   b. Biosynthesis of proteins.

   c. The role and metabolism of carnitine.

   d. Foetal protein.

2. *General Topics*

   a. Biochemical: (1) amino acids, (2) polypeptides, (3) proteins: lipo-, glyco- and hetero-proteins, proteins with specific activity in connection with coagulation, immunity and hormonal activity.

   b. Medical: (1) diagnosis – by means of analysis of proteins – in blood, urine and C.S.F., (2) therapy: correct diet.

   c. Technical: (1) physicochemical methods such as: chromatography, polarography and electrophoresis, (2) microbiological methods.

Subjects for Round Table Discussions

1. Technical advances in the analysis of proteins and related substances.
2. Advances in the study of pathological conditions connected with proteins, polypeptides and amino acids.

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